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The Effects Of Perceived Predation Risk On The Avian Brain

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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The Effects Of Perceived Predation Risk On The Avian Brain

(Thesis format: Integrated Article)

Emma Caroline Hobbs

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Predators do not affect prey solely through direct killing. The fear (i.e. the prospect of imminent, violent death) of predators shapes prey ecology– the mere presence of a predator leaves lasting effects. Current models of fear are based on post-traumatic stress disorder (PTSD) in humans. The scientific community has identified brain regions involved in mammalian fear processing. The neurobiological effects of predator fear on wild animals are unknown. I exposed wild black-capped chickadees (*Poecile atricapillus*) to auditory playbacks simulating acute and chronic predation risk and quantified the expression of short- and long-term immediate-early genes in brain regions implicated in the avian fear network: the nucleus taeniae of the amygdala (TnA), hippocampus (Hp), and caudal nidopallium (NC). The TnA and Hp showed short- and long-term changes in response to predation risk. NC results were ambiguous. I provide new information to be incorporated into the biomedical model of fear and the field of predator-prey ecology.

Keywords

Predator-prey ecology, fear, avian neurobiology, post-traumatic stress disorder, nucleus taeniae of the amygdala, hippocampus, caudal nidopallium, immediate-early genes, ZENK, *c-fos*, Δ FosB, *Poecile atricapillus*

Co-Authorship Statement

Dr. Scott MacDougall-Shackleton will be the second co-author on the manuscripts to be published from this thesis. Scott provided his expertise with regards to the capture of my study species, surgeries, and particularly in the design and implementation of my immunohistochemistry protocols and the microscopy required in order to obtain my results. His animal use protocols also allowed me to carry out this research.

Dr. Michael Clinchy will be the third co-author on the manuscripts to be published from this thesis. Mike provided his guidance in the design of my overall experiments, particularly in relation to the auditory playback treatments and data analysis. He provided valuable background knowledge about predator-prey ecology and the neurobiology of fear, in addition to essential feedback on my research as a whole.

Dr. Liana Zanette will be the fourth co-author on the manuscripts to be published from this thesis. Liana provided a great deal of knowledge that I required to design and carry out my studies. She helped develop my experimental protocols, provided feedback on data analysis and the development of my manuscripts. Her NSERC grants supported the studies required for the completion of this thesis.

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Chapter 1

General Introduction

1.1 Perceived predation risk

Predators have traditionally been seen as direct killers, reducing prey populations through consumption alone (Abrams 1993; Preisser *et al.* 2005; Schmitz *et al.* 2008). Models of predator-prey dynamics have most often focused on predators, while treating prey as unresponsive victims of predation (Murdoch & Oaten 1975; Taylor 1984; Turchin 2003). We now know that predators impact prey not only by how many they kill, but also by how many they threaten. The presence, and resulting perceived risk, of predators has far greater impacts on prey and ecosystems than direct predation alone (Schmitz *et al.* 2008). These non-consumptive predator effects have profound effects on prey by altering their physiology, behavior, and morphology.

The success rate of predators in the wild is not often considered in studies of predator-prey dynamics, despite extremely low rates of prey capture by predators across taxa (Vermeij 1982). As predators have limited success when catching prey, prey often survive these ‘near-miss’ predator attacks (McLaughlin *et al.* 2000; Cresswell & Quinn 2010; Combes *et al.* 2013). For example, it is estimated that sharks fail to kill bottlenose dolphins (*Tursiops aduncus*) at least 11% of the time after they have successfully bitten the dolphin (Heithaus 2001). Very few species have capture rates that approach even 90%, and may not kill all prey they successfully capture (Vermeij 1982). The fact that predators do not kill all prey they pursue is clarified further by the fact that up to 70% of living individuals in some prey species have scars from non-lethal predator attacks (McLaughlin *et al.* 2000; Heithaus 2001; Cresswell & Quinn 2010; Combes *et al.* 2013).

To illustrate, 13% of giraffes (*Giraffa camelopardalis*) over one year of age exhibited claw marks on their hindquarters from non-lethal lion (*Panthera leo*) attacks (Strauss & Packer 2013). We should expect that these life-threatening predator confrontations would have substantial, lasting impacts on all aspects of prey ecology. The ability of animals to recognize and respond to predation risk and avoid predator attack forms the foundation on which non-consumptive predator effects change prey populations and communities (Lima & Steury 2005). In order to act appropriately based on the risk of predation in their environments, prey use anti-predator tactics to decrease their conspicuousness and to improve their chances of escaping attack (Lima & Dill 1990; Brown *et al.* 1999; Nelson *et al.* 2004; Caro 2005).

1.2 Anti-predator responses

Anti-predator responses can be behavioural, aimed at allowing prey to better conceal themselves or to enable them to detect predators more successfully (Abrams 1986; Lima & Dill 1990; Schmitz *et al.* 2008). Prey may increase their use of refuges or decrease their feeding, parenting, or mating activity, rendering them less conspicuous (Lima & Dill 1990; Abrams 1993; Brown & Kotler 2004; Creel *et al.* 2005; Schmitz *et al.* 2008). For example, when faced with increased densities of tiger sharks (*Galeocerdo cuvier*), bottlenose dolphins abandoned highly profitable, yet risky, shallow foraging habitats to forage in less profitable, but safe deep habitat (Heithaus & Dill 2006). In addition to behavioural alteration, the physiology of prey including glucocorticoid levels, metabolic rates, and oxidative stress can all be impacted by an increase in perceived predation risk (Hik *et al.* 2001; Apfelbach *et al.* 2005; Slos & Stoks 2008; Sheriff *et al.* 2009; Hawlena & Schmitz 2010). These effects are costly: increased glucocorticoid

levels can reduce the function of gonadotropins, affecting reproduction (Sheriff *et al.* 2009); increased metabolism can result in greater energy use and increased need for food (Chabot *et al.* 1996); and oxidative stress can reduce body condition, immune function, reproduction, and survival (Janssens & Stoks 2014). These changes in behaviour and physiology were thought to be solely acute, occurring during and immediately after a near-lethal encounter with a predator, and quickly dissipating, returning the individual to its previous state. Recently, however, studies have shown that these anti-predator responses can also endure, affecting reproduction and survival (Preisser & Bolnick 2008; Anson *et al.* 2013). Elk (*Cervus canadensis*) in the Yellowstone National Park population showed a significant decline in calf recruitment in the years following the reintroduction of wolves (*Canis lupus*) to the park, despite the fact that wolves rarely kill calves, suggesting that perceived predation risk alone resulted in these declines (Creel *et al.* 2007). Zannette *et al.* (2011) found a 40% decrease in song sparrow (*Melospiza melodia*) offspring production in response to high perceived predation risk in the absence of direct predation.

Increased predation risk can also result in enduring changes in prey morphology through developmental phenotypic plasticity. In invertebrates, fish, and some amphibians, these developmental changes take the form of inducible morphological defences, alterations in body form that assist prey in surviving predator attacks (Preisser *et al.* 2005; Preisser & Bolnick 2008). The classic example of an inducible morphological defence is the spiny “helmet” that *Daphnia pulex* develop in response to increased predation risk (Krueger & Dodson 1981); other organisms develop spikes or tougher shells, for example (Bernard 2004; Orr *et al.* 2010). Terrestrial vertebrates seldom induce

external morphological defences in response to an increase in predation risk, but it has been proposed that analogous defences are predator-induced changes in neurobiology (Kavaliers & Choleris 2001; Apfelbach *et al.* 2005; Sheriff *et al.* 2009; Clinchy *et al.* 2010, 2013). These changes can affect neurotransmitters, neuroarchitecture, plasticity, and gene expression, and can persist over the long term (Slos & Stoks 2008; Zoladz *et al.* 2008, 2012; Clinchy *et al.* 2010, 2013; Cohen *et al.* 2012). Most information about how the brain responds to threat come from studies of post-traumatic stress disorder (PTSD) in humans. Results of this research demonstrate that fear (i.e., the prospect of imminent, violent death due to perceived predation risk) has profound impacts on brain structure and function over the long-term.

1.3 Fear and post-traumatic stress disorder (PTSD)

Fear is a natural, evolutionary response to threats in the environment. It results in physiological responses that support defensive behaviours (i.e., anti-predator behaviours; fighting, fleeing, remaining motionless) (Schmitz *et al.* 1997; Nelson *et al.* 2004; Macleod *et al.* 2014). These physiological and defensive responses are a means by which an organism can increase its chances of survival in the face of a threat to its life. An organism can respond innately to, or learn through experience about, a variety of possible threats (i.e., predators, social behaviours, pain) and determine how best to respond to these threats in order to survive. In this way, fear is beneficial, as it allows an individual to respond to a threat to its survival (Boonstra 2013). However, responding to fear can be detrimental to an organism's other functions, like feeding or reproduction, if like in PTSD in humans, the organism responds in fear to innocuous stimuli that it associates

with a previous life-threatening event or if the fear response far outlasts the actual threat (Shiromani *et al.* 2009).

PTSD is a chronic, incapacitating disorder that results from a traumatic experience in which one perceives a potential loss of life (Shiromani *et al.* 2009; Cohen *et al.* 2012). PTSD is characterized by extreme fear caused by the initial traumatic event, repeated re-experiencing of this event, avoidance of cues related to the trauma, and hyperarousal and hypervigilance for at least one month after the event— although most patients experience these symptoms for much longer (Shiromani *et al.* 2009). These behavioural and psychological symptoms occur in tandem with changes in physiology. Patients with PTSD show increased sensitivity in the negative feedback system of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in abnormal glucocorticoid levels (Yehuda 2002; Shiromani *et al.* 2009; Chattarji *et al.* 2015). Cortisol levels in PTSD patients have been found to be below normal for decades after the traumatic event, despite high levels of corticotropin-releasing factor in cerebrospinal fluid (Yehuda 2002). Patients also have increased circulating norepinephrine and thyroid hormones, which contribute to behavioural symptoms (e.g. hypervigilance) (Yehuda 2002).

Fear also has dramatic impacts on the function of the mammalian brain, and this fear is something that can be measured. Different types of fear— fear of pain, fear of aggressive conspecifics, and fear of predators— are processed in distinct pathways (Gross & Canteras 2012). In the study of PTSD in humans and fear in other mammals, three brain regions are commonly implicated in the processing of this fear: the amygdala, the hippocampus, and the prefrontal cortex (Shin *et al.* 2006; Sotres-Bayon *et al.* 2006; Shiromani *et al.* 2009; Gross & Canteras 2012; Chattarji *et al.* 2015).

Although the amygdala is involved in many functions, its critical role in the processing of fear is unambiguous and ubiquitous in mammalian species (Shiromani *et al.* 2009). In the brains of normal humans, the amygdala is active in response to fearful faces and aversive stimuli, providing a protective function by alerting individuals to relevant threats or cues of threat in their environments, and by processing these threats (Shiromani *et al.* 2009). In PTSD patients, however, the amygdala is hyperresponsive to both trauma-related stimuli (e.g. combat sounds) (Liberzon *et al.* 1999; Protopopescu *et al.* 2005; Shin *et al.* 2006) and innocuous stimuli associated with the event (e.g. locations, people) (Yehuda 2002). Although few studies have investigated amygdala structure in PTSD patients, some have found decreased amygdala volume in those exhibiting symptoms (Rogers *et al.* 2009). The amygdala has projections to the hippocampus and prefrontal cortex, and regulates the response to stress in each of these regions (Kilpatrick & Cahill 2003; Akirav & Maroun 2007; Shiromani *et al.* 2009; Chattarji *et al.* 2015).

The role of the hippocampus is more diverse than that of the amygdala; it is involved in the formation of memory, learning, and the processing of spatial information (Shiromani *et al.* 2009). However, the hippocampus is also involved specifically in fear processing, as evidence suggests a role for the hippocampus in the processing of spatial information to do with fear, the formation of fear memories, and the extinction of fear responses (Kim & Diamond 2002; Shin *et al.* 2006; Cornwell *et al.* 2012; Gross & Canteras 2012; Wang *et al.* 2013; Wotjak & Pape 2013). The hippocampus communicates with the amygdala and feedback moves bidirectionally between these regions (Shiromani *et al.* 2009; Chattarji *et al.* 2015). Similarly to the amygdala, studies have found decreased hippocampal volume in patients with PTSD (Shin *et al.* 2006;

Chattarji *et al.* 2015), and it has been suggested that these abnormalities may relate to memory and cognitive deficits seen in these patients, and may in fact be a risk factor for the disorder itself (Shiromani *et al.* 2009). Unlike the amygdala, which shows heightened activity in PTSD, hippocampal activity has been found to be lower than normal in PTSD patients (Schuff *et al.* 2001). The hippocampus plays an important regulatory role with regards to the HPA axis; decreased hippocampal function results in less HPA axis inhibition and consequently greater activation in this axis, leading to an increased stress response (Shiromani *et al.* 2009; Chattarji *et al.* 2015).

The prefrontal cortex is the third brain region implicated in the processing of fear, although, like the hippocampus, it has various functions (Gross & Canteras 2012; Chattarji *et al.* 2015). The primary role of the prefrontal cortex is in executive control including decision-making, as well as in the formation of fearful memories (Shin *et al.* 2006; Akirav & Maroun 2007; Maroun 2012). It has been shown to regulate the stress response by providing “top-down” control to the amygdala, inhibiting the amygdala fear response under normal circumstances (Quirk & Beer 2006; Sotres-Bayon *et al.* 2006; Akirav & Maroun 2007; Shiromani *et al.* 2009; Chattarji *et al.* 2015). However, recent studies suggest that in PTSD, the prefrontal cortex is hyporesponsive, leading to uninhibited amygdala activity; this diminished activity is one of the most consistent findings in the PTSD literature (Cerqueira *et al.* 2007; Shiromani *et al.* 2009; Chattarji *et al.* 2015).

Taken together, these three brain regions and the lasting changes in their structure and function following a traumatic experience are crucial to our understanding of the network processing fear in the human brain, and the homologous network in the brains of

other mammals. Using mammalian models in the lab, researchers have been able to delve deeper into this brain network to gain further understanding of its function and response to trauma, and to attempt to model a PTSD-like disorder (Adamec & Shallow 1993; Wiedenmayer 2004; Cohen *et al.* 2012). Initially, researchers undertaking mammalian laboratory studies of fear used aversive stimuli like restraint, foot shock, swimming stress, or changes in social hierarchy to induce a stress response in the study organisms (Clinchy *et al.* 2010). However, recent research has focused on the use of auditory and olfactory predator cues. These are perceived by rats and mice in the lab as life-threatening, but cause them no pain, best mimicking PTSD-eliciting stimuli (Adamec & Shallow 1993; Staples *et al.* 2005, 2009; Mackenzie *et al.* 2010; Cohen *et al.* 2012). The exposure of mammals in the lab to predator cues is a powerful means by which to investigate the effects of fear on the brain and has also resulted in numerous sustained behavioural, physiological, and neurobiological changes similar to symptoms of human PTSD (Wiedenmayer 2004; Zoladz *et al.* 2008, 2012; Mitra *et al.* 2009; Clay *et al.* 2011). These fear-induced changes include effects on anxiety, hormone levels, and gene expression in the brain (Adamec & Shallow 1993; Adamec *et al.* 2004; Staples *et al.* 2005; Costantini *et al.* 2010; Clinchy *et al.* 2011). Like in PTSD, a single, traumatic exposure to a threatening cue can result in lasting changes in lab mammals for weeks or months (Adamec & Shallow 1993; Adamec *et al.* 2004; Clinchy *et al.* 2010; Wotjak & Pape 2013).

1.4 Effects of fear on the brains of wild animals

It is evident that the brains of humans and other mammals in the laboratory are changed by fear, however, laboratory studies often do not translate well to wild animals

living under natural conditions (Creel & Christianson 2008). Recent studies have shown that captive raised animals may be less responsive to aversive stimuli than wild animals (Wiedenmayer 2004). Little study has gone into what the neurobiological effects of real-world predation threat are on wild, free living animals, especially non-mammalian species. Very little is known about the avian brain regions and networks processing predator fear, and no investigation into the long-term activation of the avian brain in response to predator threat has occurred. It should be expected, however, that an encounter with a predator (or a simulation of such an encounter with an auditory or olfactory cue) would be perceived as life threatening by prey, and that this type of traumatic event should result in lasting changes in the brain, as seen in humans with PTSD and mammalian lab models (Clinchy *et al.* 2010; Cohen *et al.* 2012; Boonstra 2013). The life-long predator fear that wild animals experience is more intense than any simulation that could be carried out in a laboratory setting, and therefore should result in extreme impacts on their neurobiology.

The quantification of the impacts of predator-induced fear on wild animals is additionally a more meaningful metric than measuring this same fear in lab mammals. Because wild animals have almost certainly experienced predator threat in their environment, they are likely functioning at a fear level higher than the baseline that would be expected of a predator-naïve lab model. As a result, any significant increases in any measure of fear (behavioural, physiological, or neurobiological) in a wild animal represent a meaningful impact of fear (Creel & Christianson 2008; Clinchy *et al.* 2010, 2013; Cohen *et al.* 2012).

Three avian brain regions have been suggested as parts of the network processing

predator-induced fear in the avian brain: the nucleus taeniae of the amygdala (TnA), the hippocampus (Hp), and the caudal nidopallium (NC). The nucleus taeniae of the amygdala (TnA) is known to be the avian homologue of the medial amygdala, and is proposed to be the avian fear centre (Cohen & Goff 1978; Charlier *et al.* 2005). It has been proposed that this region, like its mammalian counterpart, acts as a switchboard, gathering information about potential threats in the environment and routing this information to other areas of the brain for processing. Previous studies have shown the TnA and its mammalian homologue to be activated in response to aversive stimuli, such as foot shock, as well as to unambiguous cues of predation threat including predator mounts and, in the case of the mammalian amygdala, olfactory predator cues (Dielenberg *et al.* 2001; Li *et al.* 2004; Brito *et al.* 2011; Marzluff *et al.* 2012; Cross *et al.* 2013).

The proposed role of the avian hippocampus (Hp), homologue of the mammalian hippocampus, in the processing of predator-induced fear is more ambiguous than that of the TnA; the Hp has been implicated in many processes, including several fear-related functions such as the formation of memory of fearful stimuli and processing of spatial and social information (Clayton & Lee 1998; Colombo & Broadbent 2000; Kim & Diamond 2002; Mayer *et al.* 2010; Nishizawa *et al.* 2011; Cornwell *et al.* 2012; Cross *et al.* 2013).

The role of the NC in the processing of predator-induced fear has thus far been ambiguously described, as it is involved in many processes. The NC is analogous to the mammalian prefrontal cortex, and it has been proposed that it is involved in executive function and decision-making (Veit & Nieder 2013), and has previously been found to be active in response to aversive and fearful stimuli (Rose & Colombo 2005; Cross *et al.*

2013) (Figure 1.1).

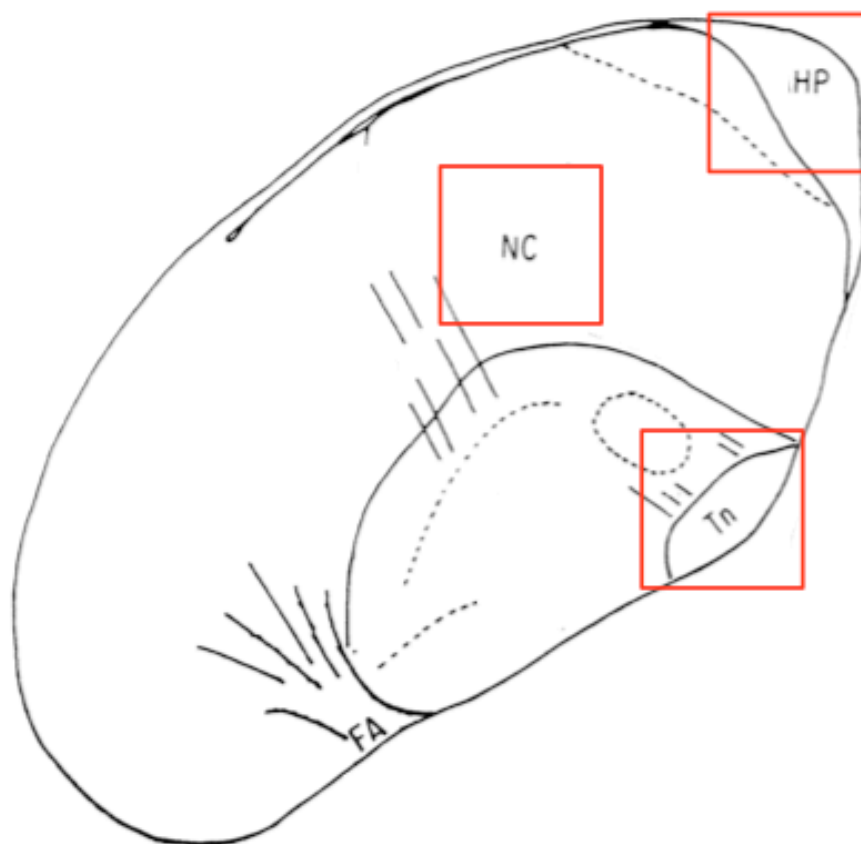


Figure 1.1. Regions proposed to play a role in the processing of fear in the avian brain: the nucleus taeniae of the amygdala (TnA), the hippocampus (Hp), and the caudal nidopallium (NC) as viewed in one hemisphere of a coronal brain slice. Locations of brain regions are indicated by labels and red outlines.

1.5 Measuring fear in the brain

One of the major obstacles to studying the effects of perceived predation risk on the brain is how we measure this fear (Lima & Dill 1990). In order to measure changes in activation in the brain, the protein products of immediate-early genes (IEGs) can be labelled and quantified using immunohistochemistry. Two main IEGs have been used

previously to investigate activation in the avian and mammalian brains, ZENK (Kimpo & Doupe 1997; Bailey & Wade 2003; Phillimore *et al.* 2003; Knapska & Kaczmarek 2004; Charlier *et al.* 2005; Leitner *et al.* 2005; Avey *et al.* 2008; Mayer *et al.* 2010; Brito *et al.* 2011) and *c-fos* (Kimpo & Doupe 1997; Dielenberg *et al.* 2001; Wiedenmayer & Barr 2001; Charlier *et al.* 2005; Staples *et al.* 2005; Cunningham *et al.* 2008; Vanelzakker *et al.* 2011). These IEGs are used as short-term markers of brain activation, as the protein products of these genes are produced and degraded in active neurons within hours of a stimulus exposure (Cole *et al.* 1989; Kimpo & Doupe 1997; Guzowski *et al.* 2001; Thiriet *et al.* 2001; Mokin & Keifer 2005).

ZENK is a gene encoding a nuclear transcription factor protein, ZENK, which is rapidly induced following exposure to an extracellular stimulus. ZENK protein binds to DNA and activates transcription of target genes, protein products of which are required for cell division and differentiation. ZENK is not produced in all neuron populations, but cells expressing ZENK protein in their nuclei are considered activated (Cole *et al.* 1989; Guzowski *et al.* 2001; Thiriet *et al.* 2001; Mokin & Keifer 2005). The immediate-early gene *c-fos* encodes the *c-fos* protein, which is rapidly translated and acts as a transcriptional regulator for several target genes. Like ZENK, *c-fos* is not expressed in all neurons, but when it is, this is an indication that this cell has been activated by an external stimulus (Guzowski *et al.* 2001; Thiriet *et al.* 2001; Mokin & Keifer 2005).

In order to measure long-lasting changes in activity in the mammalian brain, researchers have used the IEG FosB. The protein product of this gene, FosB, is a transcription factor that is induced by chronic external stimuli. A splice variant of the FosB protein, Δ FosB, is unusually stable and can persist in the cell for weeks or months,

acting as a transcriptional regulator, influencing plasticity and behaviour (McClung *et al.* 2004; Nestler 2008). Δ FosB is present in active mammalian neurons for at least a week following chronic stimulus exposure. Labelling Δ FosB over the weeks post-stimulus provides a picture of lasting activation as a result of the stimulus (McClung *et al.* 2004; Nestler 2008). Despite its use in mammalian studies, Δ FosB has never before been labelled in the avian brain. It is unknown whether it is produced or can be labelled in the neurons of birds.

1.6 The importance of wild animals

The vast majority of research into the effects of fear on the brain has so far taken place in a biomedical context with controlled stimuli and human patients or model mammalian subjects. It is unknown whether predator-induced fear results in similar changes in the brains of wild animals, especially non-mammals, exposed to constant and unpredictable predation threat in their environments. Wild animals were not considered in fear research until recently; they were previously thought to be unaffected by predator fear in the long term, as it was thought to be maladaptive for a free-living animal to be debilitated by stress (Sapolsky 2004). Fear of a predator was considered an acute stress response immediately following a predator encounter and quickly dissipating (Krebs 2002). However, studies have shown that fear effects on free-living animals result in lasting changes in behaviour and reproduction (Wiedenmayer 2004; Creel & Christianson 2008; Hawlena & Schmitz 2010); for example, by changing foraging activity (Brown & Kotler 2004; Heithaus & Dill 2006; Zanette *et al.* 2013), habitat selection (Creel *et al.* 2005; Eggers *et al.* 2006), and reproduction (Eggers *et al.* 2006; Zanette *et al.* 2006, 2011; Creel *et al.* 2007; Travers *et al.* 2010). The exposure of wild animals to predator

cues may prove more advantageous for the study of PTSD than mammalian lab models (Clinchy *et al.* 2010, 2013; Cohen *et al.* 2012). The threat of predation in the real world is not an occasional or predictable one. Wild animals could have a life-threatening encounter with a predator at any time, every day of their lives. Predator-induced changes in behaviour, physiology, and neurobiology in response to persistent and unpredictable predator-induced fear in the wild are easily related to PTSD. This fear is a valid, ethologically relevant experience for wild animals, and the resulting effects may mimic those seen in PTSD patients to an even greater degree than the dramatic effects already demonstrated in the lab (Wiedenmayer 2004). Thus, the study of fear in wild animals is crucial to further clarify how life-threatening experiences affect behaviour, physiology, and neurobiology, and will provide biomedical researchers with new information about the causes and symptoms of PTSD in humans.

In addition to the biomedical applications of the study of fear on the brains of wild animals, this research is the first step to linking changes in the brains of wild animals to changes in their behaviour and physiology, and the effects of these changes on reproduction and population dynamics. We now know that the fear of predators has impacts on foraging, reproduction, and parental care, with effects spanning generations (Creel *et al.* 2005, 2007; Eggers *et al.* 2006; Zanette *et al.* 2006, 2011, 2012, 2013; Travers *et al.* 2010), and results in altered physiology (Clinchy *et al.* 2004; Creel *et al.* 2007; Hawlena & Schmitz 2010; Newman *et al.* 2012; Zanette *et al.* 2012). These changes in turn can lead to decreased survival and fecundity in prey species, having population level effects and changing prey demography. However, no connection has been made between these behavioural and physiological changes and altered brain

activity. We should expect, based on links between changes in the brain and changes in behaviour seen in PTSD, that similar connections will be found in studies of wild animals. The first step to making these important links is the investigation of how fear is changing the brains of these animals, and later investigating parallel changes in behaviour and physiology.

1.7 Research goals

We know that life-threatening events change the brains of humans, and that the brains of laboratory mammals are affected similarly in response to simulations of predator threat, both in the short- and long-term. It is unknown whether wild animals are affected to the same degree as those raised in captivity, and the networks processing predator fear in the brains of non-mammalian taxa are not yet well understood. No experiment has tested the acute and lasting effects of predator stimuli on wild-caught non-mammals.

In Chapter 2, I address three brain regions of interest in avian fear processing in black-capped chickadees (*Poecile atricapillus*), and investigate acute and lasting activation changes in these regions in response to short-term and chronic simulations of predation risk. In Chapter 3, I investigate short-term activation changes in the same chickadee brain regions in response to black-capped chickadee alarm calls, which act as social cues of different degrees of predation threat. In Chapter 4, I discuss the broader implications of my findings and their application to our biomedical understanding of how fear impacts the brain and to predator-prey ecology. I also suggest aspects of predator-prey neurobiology that merit further investigation.

1.8 Study species

Black-capped chickadees are one of the most recognizable birds in North America. Their range covers almost all of Canada and most of the United States (all general life history of chickadees reviewed here is from Smith 1991, unless noted otherwise). Chickadees are resident year round, making them ideal research subjects, as they are accessible in any season. Chickadees, ranging in mass from 10 to 14 g, are characterized by their dark cap and bib, white cheeks, and dark back. They feed frequently and cache food for later use in multiple locations.

Chickadees live in nonbreeding flocks in the fall and winter, and defend territories in monogamous breeding pairs in the spring and summer. Both sexes excavate the cavities in which they build and incubate their nests, but females alone construct the nest and incubate the eggs. Common predators of chickadees include sharp-shinned (*Accipiter striatus*) and Cooper's hawks (*Accipiter cooperii*), and commonly in the area in which I conducted my research, northern saw-whet owls (*Aegolius acadicus*).

Chickadees have a complex social system consisting of an extensive vocal repertoire of at least 11 distinct vocalizations, encoding different messages. When confronted with a perceived threat, chickadees use one of two vocalizations to alert conspecifics. In the case of a moderately threatening predator, chickadees use the *mobbing call*. This call is used by both sexes to alert other flock members to a potential threat, calling the flock together to confront, or mob the threat. An example of a moderately alarming predator is a stationary avian predator within view of at least one member of the flock. In the case of extreme alarm, such as a flying avian predator or a predator that no individual has localized, chickadees use a *high zee call*. Only males emit

this high frequency call. The call alerts flock members to the threat and induces immobility until the threat is no longer imminent.

The chickadees used in my study are resident to the area of London, ON around the University of Western Ontario year round. Chickadees approach feeders easily, making them easy to capture in Potter traps for study, although capture is easier in fall and winter when chickadees flock together and food is scarce. Wild-caught chickadees are known acclimatize well to captivity, and the chickadees I used did well in semi-natural outdoor aviaries on campus, close to where they were captured.

1.9 References

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Chapter 2

2.1 Introduction

All organisms face the threat of predator attack. How individuals respond to these threats impacts all aspects of ecology, from individual physiology to population dynamics. Understanding the mechanisms by which prey process these life-threatening encounters is essential to our knowledge of predator-prey ecology and has broad implications for the study of human anxiety disorders.

Research regarding the effects of predators on their prey has traditionally focused on direct predation (i.e., predators killing prey for food) and, more recently, on the short-term effects of non-lethal predation events (i.e., perceived predation risk). For example, increased predation risk can cause prey to intensify anti-predator behaviours in order to become less conspicuous in the presence of predators (Lima 1998; Schmitz *et al.* 2008). Perceived predation risk and predator encounters can also impact physiology (Clinchy *et al.* 2004) including glucocorticoids (Hik *et al.* 2001; Creel *et al.* 2007; Newman *et al.* 2012) and measures of oxidative stress (Slos & Stoks 2008; Travers *et al.* 2010). However, we now know that these acute changes in behaviour and physiology occur in tandem with *lasting* indirect predator effects— persistent changes in behaviour, physiology, and morphology resulting from a perceived predation threat or an actual predation attempt (Preisser *et al.* 2005; Creel & Christianson 2008; Preisser & Bolnick 2008; Ferrari 2014). In fish, amphibians, and invertebrates, increased threat of predation can cause organisms to induce morphological defences in order to protect against potential attack (Bernard 2004; Orr *et al.* 2010), a classic example being the protective

‘helmet’ that *Daphnia pulex* develop in response to increased predation pressure (Krueger & Dodson 1981).

In terrestrial vertebrates, which seldom develop induced external morphological defences in response to increased predation risk, predator-induced defences may take the form of changes in the morphology and activity of neurons in the brain, most examples of which come from studies of post-traumatic stress disorder (PTSD) in humans (Shiromani *et al.* 2009; Orr *et al.* 2010; Cohen *et al.* 2012; Clinchy *et al.* 2013). As a result of research into the causes and symptoms of PTSD, it is now possible to measure some impacts of fear (i.e., the prospect of imminent, violent death due to perceived predation risk) on the brains of prey, as well as to demonstrate that the neurobiological effects of predator-induced fear in prey persist over time.

Humans exposed to a fearful, seemingly inescapable, and life-threatening stimulus are at risk of developing PTSD (Shiromani *et al.* 2009; Zoladz *et al.* 2012; Chattarji *et al.* 2015). After as little as one brief, traumatic experience, the resulting changes in behaviour, physiology, and neurobiology are immediate and can be life-long (Yehuda 2002; Wiedenmayer 2004; Shiromani *et al.* 2009; Chattarji *et al.* 2015) (Chapter 1). Until recently, most research into fear and its effects has been biomedical, stemming from our interest in PTSD and other fear-related disorders. The aim of this biomedical research has traditionally been to investigate the effects of chronic *psychological* stress in humans, whereas ecological research has traditionally focused on the acute *physiological* crisis of prey surviving a predator attack (Clinchy *et al.* 2013). Recently, however, small mammalian models have been used to further investigate the causes and symptoms of PTSD in a laboratory context (Adamec & Shallow 1993; Wiedenmayer 2004; Cohen *et*

al. 2012). Predator cues are reliable indicators of a possible attack and thus are perceived as life-threatening– yet are painless– fitting the aetiology of PTSD better than any stimuli previously used (Adamec & Shallow 1993; Staples *et al.* 2005, 2009; Mackenzie *et al.* 2010; Cohen *et al.* 2012). Exposure to these predator cues could result in “psychological stress” or “fear” similar to PTSD-inducing fear in humans (Adamec *et al.* 2004; Clinchy *et al.* 2013) and has resulted in numerous sustained changes in behaviour (Adamec *et al.* 2004; Mackenzie *et al.* 2010; Staples 2010), physiology (Clay *et al.* 2011), and neurobiology (Mitra *et al.* 2009) (Chapter 1). In studying predator-induced fear and the similar fear of losing one’s life leading to PTSD, researchers have identified which parts of the mammalian brain are active in response to fearful stimuli (including fear of predators), and often focus on three main regions: the medial amygdala, the hippocampus, and the prefrontal cortex (Rosen & Schulkin 1998; Kilpatrick & Cahill 2003; Phelps 2004; Shin *et al.* 2006; Shiromani *et al.* 2009; Clinchy *et al.* 2013; Chattarji *et al.* 2015).

The amygdala is the region most unequivocally implicated in the processing of all fearful stimuli and is thought to be central to the network processing fear in the mammalian brain, acting as a switchboard that collects information about environmental threats and directs them into the complex and distinct efferent pathways for the fear of pain, conspecifics, or predators (Cohen & Goff 1978; Shin *et al.* 2006; Gross & Canteras 2012; Chattarji *et al.* 2015). The hippocampus is a crucial component of the predator-induced fear pathway, collecting spatial and contextual information from the environment (Cornwell *et al.* 2012) and forming memories of fearful experiences (Kim & Diamond 2002; Wang *et al.* 2012) among other functions. The prefrontal cortex has various

functions, some of which are related to the processing of fearful stimuli. It gathers information about perceived threats and allows individuals to make decisions and respond appropriately to fearful stimuli (Shin *et al.* 2006; Akirav & Maroun 2007; Shiromani *et al.* 2009; Gross & Canteras 2012; Maroun 2012). All of these regions have been shown to be active over the short- and long-term in response to life-threatening fear or predation threat in humans and mammalian models (Shiromani *et al.* 2009; Mackenzie *et al.* 2010; Martinez *et al.* 2011; Wang *et al.* 2013; Chattarji *et al.* 2015). They are connected in a feedback network with bidirectional communication among the regions (Chattarji *et al.* 2015) (Chapter 1).

Predator-induced fear was not thought to dramatically affect animals in the wild until recently, when studies showed that fear effects on free-living animals result in lasting changes in behaviour and reproduction (Kavaliers & Choleris 2001; Wiedenmayer 2004; Creel & Christianson 2008; Hawlena & Schmitz 2010; Zanette *et al.* 2011). It is unknown whether the brains of other taxa are altered by the experience of fear in the wild, which brain regions are impacted, and if these changes are quantifiable. The dramatic behavioural effects of predator-induced fear in wild mammals have led researchers to suggest that wild animals exposed to predator cues will be more advantageous for the study of PTSD than lab models (Clinchy *et al.* 2010, 2013; Cohen *et al.* 2012). The ability to measure the effects of fear in wild animals previously living in their natural environment with their predators is powerful (Creel & Christianson 2008), as these wild animals—constantly vulnerable to the possibility of predator attack—already function above the physiological and behavioural baseline of predator-naïve, lab-raised animals (Boonstra 2013). Dramatic changes in response to experimental predator

exposure in animals likely already affected by predators in their natural environments would represent strong impacts of predators.

Three avian brain regions have been proposed to function in a network similar to that processing fear in the mammalian brain. The nucleus taeniae of the amygdala (TnA) is the avian homologue of the medial amygdala, and is proposed to be the centre of the avian fear network (Cohen & Goff 1978; Charlier *et al.* 2005; Marzluff *et al.* 2012). The avian hippocampus (Hp) is homologous to its mammalian counterpart and is involved in the formation of memory, including fear memory, and the processing of spatial and social information (Sherry & Duff 1996; Clayton & Lee 1998; Mayer *et al.* 2010; Nishizawa *et al.* 2011; Cornwell *et al.* 2012; Cross *et al.* 2013). Finally, the avian caudal nidopallium (NC) acts analogously to the mammalian prefrontal cortex, and is involved in aspects of executive function, such as decision making, allowing individuals to respond to a fearful stimulus (i.e., a predator mount) (Rose & Colombo 2005; Cross *et al.* 2013) (Chapter 1). Our knowledge of the function of these brain regions in relation to predator-induced fear is thus far minimal, and no investigation into their long-term activation in response to predator threat has occurred. It should be expected, however, that an encounter with a predator (or a simulation of such an encounter with an predator cue) would be perceived as life threatening by individual prey. This type of traumatic event should result in acute and lasting changes in the brain, as seen in humans with PTSD and mammalian lab models.

As little is known about the regions processing predator-induced fear in the avian brain and what long-term effects, if any, this fear produces in a wild animal, my study sought to investigate short- and long-term changes in avian brain activation in a

simulation of predation risk. First, I used auditory predator playbacks to examine short-term changes in the TnA, Hp, and NC of wild-caught black-capped chickadees (*Poecile atricapillus*), using two short-term immediate-early gene (IEG) immunohistochemical assays (ZENK and *c-fos*) to label active neurons. Next, I investigated long-term changes in activation in the same three brain regions in response to an auditory simulation of chronic predation threat using the IEG FosB, and labelling its protein splice variant Δ FosB.

Δ FosB has previously been labelled exclusively in mammalian studies and acts as a long-term marker of brain activation. FosB is activated immediately after a stimulus and is subsequently degraded, resulting in the accumulation of Δ FosB, which plays a regulatory role in neurons for at least seven days (McClung *et al.* 2004; Nestler 2008). In order to assess the roles of these regions in fear processing and to quantify long-term changes in activation, I labelled Δ FosB four and seven days following stimulus exposure.

Δ FosB allows us to quantify lasting changes analogous to those seen in PTSD over the months following a trauma. Symptoms lasting for one month are required for a PTSD diagnosis (Shiromani *et al.* 2009). Changes in the behaviour and neurobiology of small mammals that last seven days represent similarly lasting changes to those seen in PTSD, when individual lifespan is considered; these changes are considered “long-term” (Adamec & Shallow 1993; Cohen *et al.* 2004, 2012). Based on this, changes in the brains of chickadees lasting a week also represent a long-term change, as they have similar average life spans to small lab mammals (Smith 1991), and as Δ FosB expression seven days after a stimulus exposure is considered long-term expression in the mammalian literature, and represents a lasting change in the mammalian brain (Staples *et al.* 2009; Mackenzie *et al.* 2010).

I predicted that activity in the TnA, Hp, and NC would be affected in both the short- and long-term by auditory playbacks simulating predation risk. I predicted that each of these regions would demonstrate increased activation in response to predation threat based on the proposed roles of these brain regions in fear processing. Based on previous studies which found differences in the expression of the protein products of different IEGs in response to the same stimuli, I also expected to see some differences between the two short-term IEG assays within brain regions (Sheng & Greenberg 1990; Nastiuk *et al.* 1994; Kimpo & Doupe 1997; Bock *et al.* 2005). I expected Δ FosB expression to be significantly greater at seven days post-stimulus in comparison to four days post-stimulus, as it continues to accumulate and work in the mammalian brain in the week following stimulus exposure (McClung *et al.* 2004; Nestler 2008). Here, I report that the TnA, Hp, and NC are significantly activated in comparison to the control in response to increased predation threat in the short-term, and that differences between the ZENK and *c-fos* assays were found in the NC. I also report that this increased activation in the TnA and Hp in response to increased predation risk lasts for at least a week post-stimulus, while I found no significant differences in Δ FosB expression in the NC between the treatments after seven days.

2.2 Methods

Overview

I used wild-caught black-capped chickadees (*Poecile atricapillus*) to investigate the effects of short-term and long-term perceived predation risk on three regions of the avian brain in two studies. I manipulated perceived predation risk using auditory playbacks. In my short-term study, I used the calls of a known chickadee predator, the

northern saw-whet owl (*Aegolius acadicus*) to simulate high predation risk. My non-threatening, non-predator stimuli were red-breasted nuthatch (*Sitta canadensis*) calls, which acted as my control. I used two immediate-early gene assays to quantify immunoreactivity, and thus activity, in brain regions thought to be involved in processing predator risk: the nucleus taeniae of the amygdala (TnA), the hippocampus (Hp), and the caudal nidopallium (NC).

In my long-term study, I modified protocols from the mammalian literature to simulate chronic perceived predation risk (Staples *et al.* 2009; Mackenzie *et al.* 2010) and exposed individual chickadees to predator or non-predator playbacks composed of multiple species for two days. I determined whether the TnA, Hp, and NC remained active four and seven days post-predator exposure by labelling the protein splice variant, Δ FosB, of the immediate-early gene FosB. A pilot study I conducted on six individuals resulted in high-quality Δ FosB labelling at four and seven days post-exposure in the chickadee brain.

Perceived Predation Risk Manipulations

Study One: short-term perceived predation risk manipulation

Between November 2012 and February 2013, I captured 12 black-capped chickadees (six M; six F, all after hatch year) using seed-baited Potter traps and mist nets from several sites at the University of Western Ontario in London, Ontario, Canada (43°00'37" N, 81°16'47" W). I weighed the chickadees and sexed them based on wing chord. I housed chickadees in groups of three to eight in outdoor aviaries, a semi-natural environment, for at least seven days following capture to acclimate to captivity before

manipulations began. Chickadees had access to Mazuri small bird diet (PMI Nutrition International, LLC, Brentwood, MO), black oil sunflower seeds, mealworms, and water *ad libitum*.

I randomly assigned six chickadees to each of the two playback treatments while ensuring balanced sex ratios (three M, three F per treatment). Then, I relocated chickadees to individual cages (25 cm × 30 cm × 37 cm), each within an individual sound-attenuating acoustic chamber (Industrial Acoustics Company, Inc., Bronx, NY) for 24 h before the treatment began. Each chamber was on a natural light cycle (11.5 L:12.5 D) and chickadees had access to food and water *ad libitum*. Prior to moving the chickadees, I outfitted each sound chamber with one set of speakers (Koss HDM/111BK) attached to a HipStreet (model HS-636-4GBBL) mp3 player positioned outside the chamber, allowing me to begin playback treatments without disturbing the birds. I obtained northern saw-whet owl and red-breasted nuthatch playbacks from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA). I organized vocalizations for each treatment into playlists consisting of three calls, each from a different individual (i.e., individuals a-b-c; d-e-f etc.), with each call playing for 5 s, followed by 45 s of silence. This 60 s playlist repeated 30 times at 74 dB SPL (following Avey *et al.* 2011) (Appendix B). At this time, I turned off the chamber lights and individuals remained in the silent, dark auditory chamber for 90 min before being transferred to a post-mortem room and euthanized using an overdose of isoflurane followed by transcardial perfusion with 0.1M phosphate buffered saline (PBS) (pH 7.4) and 4% paraformaldehyde (following Avey *et al.* 2011).

Study Two: long-term perceived predation risk manipulation

Between September and November 2014, I captured 24 black-capped chickadees (12 M; 12 F, all after hatch year) using seed-baited Potter traps from several sites at the University of Western Ontario in London, Ontario, Canada (43°00'37" N, 81°16'47" W). I housed chickadees in groups of three to eight in semi-natural outdoor aviaries for at least seven days following capture to acclimate to captivity. Chickadees had access to Mazuri small bird diet (PMI Nutrition International, LLC, Brentwood, MO), black oil sunflower seeds, mealworms and water *ad libitum*.

I randomly assigned twelve individuals to each of the two playback treatments, predator or non-predator, ensuring balanced sex ratios (six M, six F per treatment). I then relocated individuals to cages (25 cm × 30 cm × 37 cm) each within individual sound-attenuating acoustic chambers (Industrial Acoustics Company, Inc., Bronx, NY) for 24 h preceding their assigned playback treatment. Each chamber was on a natural light cycle (11.5 L:12.5 D) and food and water was available *ad libitum*. Chamber setup for playbacks was identical to that of the first study. Following the 24 h acclimation period, each treatment lasted for two days and individual chickadees heard either predator or non-predator sounds from sunrise to sunset. After the 48 h playback treatment, I returned each individual to its semi-natural home aviary, after which I sacrificed six individuals (three M, three F) from each treatment after four days, and six individuals from each treatment after seven days.

I matched each predator with a non-predator species for maximum amplitude and frequency using RavenLite (Version 1.0, Cornell University Lab of Ornithology:

Bioacoustics Research Program, 2010). I measured the sound level using a sound pressure metre in the centre of the cage at the height of the perches at 74 dB SPL. I obtained all calls from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA) and the Xeno-Canto foundation (www.xeno-canto.org). I used seven predator species known to prey upon chickadees (Cooper's hawk, *Accipiter cooperii*; American crow, *Corvus brachyrhynchos*; red-tailed hawk, *Buteo jamaicensis*; barred owl, *Strix varia*; sharp-shinned hawk, *Accipiter striatus*; northern saw-whet owl, *Aegolius acadicus*; merlin, *Falco columbarius*), and seven matched non-threatening non-predator species (song sparrow, *Melospiza melodia*; mallard, *Anas platyrhynchos*; blue jay, *Cyanocitta cristata*; northern leopard frog, *Lithobates pipiens*; hairy woodpecker, *Picoides villosus*; wood frog, *Lithobates sylvaticus*; downy woodpecker, *Picoides pubescens*).

I randomly selected calls from each species in a treatment group to create six unique two-hour playlists for each treatment, which consisted of 110 min of silence with 10 min of calls randomly spaced throughout. I used every species between one and four times (depending on call length) within a treatment in each playlist. I randomized the calls within periods of silence to avoid the possibility of the chickadees habituating to the playback treatments (modified from Zanette *et al.* 2011) (Appendix B).

After four or seven days in their home cage, I euthanized and perfused each individual as described in Study One.

Brain Processing

After sacrifice and perfusion, I located the gonads of all individuals in order to confirm their sex. I removed all brains and left them in 4% paraformaldehyde for a minimum of 24 h, followed by 30% sucrose for 24 h until saturated, and then I froze each brain at -80 °C for seven days. I sectioned all brains into 40 µm coronal slices using a cryostat at -20 °C. Starting with the tenth slice, I collected every tenth slice for Nissl staining until the anterior commissure was no longer visible (~ slice 120). Then I collected every slice for Nissl and three series to be used for immunohistochemistry, respectively. Nissl slices were used to locate regions of interest within the brain. For brains from the first study, I carried out immunohistochemistry for ZENK (primary antibody Egr-1 rabbit, C-19, sc-189, Santa Cruz Biotechnology) and *c-fos* (primary antibody (c-fos (4) rabbit IgG, sc-52, Santa Cruz Biotechnology) according to a standard IEG protocol at a concentration of 1:4000 and 1:500 in 0.3% phosphate-buffered saline with triton (PBS/T), respectively. For our second study, I carried out immunohistochemistry to label ΔFosB (FosB (102) rabbit IgG, sc-48, Santa Cruz Biotechnology) according to a standard IEG protocol, with the primary antibody at a concentration of 1:500 in 0.3% phosphate-buffered saline with triton (PBS/T).

I quantified immunoreactivity in the nucleus taeniae of the amygdala (TnA), hippocampus (Hp), and caudal nidopallium (NC) for each slice with the region of interest identifiable in both hemispheres. I quantified immunoreactivity in a control region in my second study (located on the most dorsal-lateral surface of each slice; to ensure that the expression I quantified in my regions of interest was meaningful and that I was not quantifying “background” expression) (Appendix A).

I captured a z-stack image of each region in each slice using a Leica CTR6500 microscope and Leica Application Suite (Leica Microsystems, Version 4.4) using 5X (Hp and NC) or 10X (TnA and control) objective lenses with the brain region centred in the field of view. I calibrated ImageJ software (Version 1.46r, National Institutes of Health, USA) to the magnification of each image and used it to measure the area of each region in mm^2 in each slice. I converted each image from colour to 16 bit, then subtracted the background and enhanced the contrast. Next, I used thresholding within ImageJ to convert IEG positive nuclei to black against a white background. I then used the counting function within ImageJ to count the IEG nuclei within each image in order to calculate IEG positive cells / mm^2 in each slice of each brain region. I captured all images and counted all cells without knowing which treatment each slice belonged to, so as not to bias the results.

Statistical Analyses

To analyse results from my first study, I counted IEG positive cells / mm^2 per slice using ImageJ software in each brain region. I averaged across all slices per brain region per individual to give me one data point per individual for each of the TnA, Hp, and NC. Then I calculated a mean per treatment within each brain region for each of the two IEGs. I then compared the mean count of IEG positive cells / mm^2 for each treatment group within each brain region using 2-factor ANOVAs with playback treatment (predator vs. non-predator) and sex as fixed factors.

For my second study, I counted ΔFosB positive cells / mm^2 per slice using ImageJ software in each brain region. I averaged across all slices per brain region per individual to give me one data point per individual for each of the TnA, Hp, NC, and control region.

I then used three-factor ANOVAs with the playback treatments (predator vs. non-predator), post-exposure day (4 d vs. 7 d), and sex as my three variables. I found no significant effects or interactions within my control region for this study, and it is therefore not included in my results. Sex was included as a fixed factor in all statistical analyses in order to rule out any possible sex effects, as most mammalian research is done exclusively on males.

In both studies, slices in which the area of interest was only present in one hemisphere due to tissue damage were excluded from calculations. Prior to parametric analyses in SPSS Statistics for Macintosh (Version 20.0; IBM Corporation, Armonk, NY, USA, 2011), data from my first study were Box-Cox transformed in order to meet the assumptions of normality and homogeneity of variances, while data from study two met these assumptions. I present non-transformed means \pm SE for clarity.

2.3 Results

Nucleus taeniae of the amygdala (TnA)

The TnA was active in response to predator calls in both the short- and long-term. There were significantly more immunoreactive cells in the predator treatment for ZENK and *c-fos* (Figs. 2.1.a,b; treatment, $F_{1,7} = 6.9$, $p = 0.034$ and $F_{1,7} = 9.4$, $p = 0.018$, respectively). I found a 73% increase in ZENK and a 94% increase in *c-fos* expression in the TnA after the chickadees had heard sounds of predators compared to the non-threatening sounds of the red-breasted nuthatch. I found no significant main effects of sex with either assay (sex, ZENK: $F_{1,7} = 0.1$, $p = 0.72$; *c-fos*: $F_{1,7} = 0.02$, $p = 0.89$), and both sexes responded similarly across the perceived predation risk treatments for the ZENK and *c-fos* assays (treatment \times sex, ZENK: $F_{1,7} = 4.5$, $p = 0.07$; *c-fos*: $F_{1,7} = 0.08$, $p = 0.78$).

The TnA showed increased long-term immunoreactivity in response to perceived predation risk (treatment, $F_{1,20} = 18.2$, $p < 0.0001$), exhibiting 70% more Δ FosB expression after one week post-exposure compared to the non-predator treatment (Fig. 2.3.a). This result was similar between four days post-exposure and seven days post-exposure (post-exposure day, $F_{1,20} = 0.6$, $p = 0.46$) with no significant interactions (treatment \times post-exposure day, $F_{1,20} = 0.765$, $p = 0.39$). The sexes, too, responded similarly to the treatments (sex, $F_{1,20} = 1.5$; $p = 0.24$) with no significant interactions with playback treatment (treatment \times sex, $F_{1,20} = 0.06$; $p = 0.82$) or with playback treatment and post-exposure day (treatment \times post-exposure day \times sex, $F_{1,20} = 1.1$; $p = 0.30$).

Hippocampus (Hp)

The hippocampus (Hp) was activated in response to increased predation risk in the short- and long-term. There were significantly more immunoreactive cells using both ZENK (Fig. 2.1.c; treatment, $F_{1,6} = 11.6$, $p = 0.014$) and *c-fos* (Fig. 2.1.d; treatment, $F_{1,7} = 14.6$, $p = 0.007$). Immediate-early gene expression was increased by up to 66% when chickadees heard the predator compared to the non-predator sounds. These effects were consistent between the sexes for ZENK (treatment \times sex, $F_{1,6} = 3.8$, $p = 0.098$; sex, $F_{1,6} = 3.7$, $p = 0.10$) and *c-fos* (treatment \times sex, $F_{1,7} = 2.5$, $p = 0.16$; sex, $F_{1,7} = 0.45$, $p = 0.52$). The Hp showed a more than 40% increase in Δ FosB expression in response to the threatening calls of predators than to the calls of non-predators for a week following stimulus exposure (Fig. 2.3.b; treatment, $F_{1,18} = 14.5$, $p = 0.001$). This result remained consistent at four days post-exposure (post-exposure day, $F_{1,19} = 0.2$, $p = 0.63$) with no interaction (treatment \times post-exposure day, $F_{1,19} = 0.7$, $p = 0.42$). The sexes too responded similarly to the two playback treatments (sex, $F_{1,19} = 1.0$; $p = 0.32$) with no

significant interactions with playback treatment (treatment \times sex, $F_{1,19} = 0.01$; $p = 0.92$) or playback treatment and post-exposure day (treatment \times post-exposure day \times sex, $F_{1,19} = 1.3$; $p = 0.27$).

Caudal nidopallium (NC)

Unlike the TnA and Hp, the caudal nidopallium (NC) showed differential activation among the playback treatments depending on which IEG was used. I found significant differences between the treatments for ZENK and *c-fos* (Fig. 2.2.a,b; treatment, $F_{1,7} = 19.2$, $p = 0.003$ and $F_{1,7} = 9.3$, $p = 0.02$, respectively) (Fig. 2). The predator treatment resulted in significantly higher ZENK expression than the control, and this effect remained consistent between the sexes (sex, $F_{1,7} = 0.07$, $p = 0.80$) with no interaction (treatment \times sex, $F_{1,7} = 0.3$, $p = 0.58$). Conversely, *c-fos* expression in the NC was significantly lower in response to the predator sounds compared to the non-predator calls. I found no main effect of sex ($F_{1,7} = 5.3$, $p = 0.06$) or treatment by sex interaction ($F_{1,7} = 0.005$, $p = 0.95$). Δ FosB expression in the NC was not significantly different when chickadees heard predator sounds in comparison to non-predator sounds (Fig. 2.3.c; treatment, $F_{1,20} = 0.9$, $p = 0.34$). This effect remained consistent between four and seven days post-exposure with no interactions (post-exposure day, $F_{1,20} = 0.3$, $p = 0.56$; treatment \times post-exposure day, $F_{1,20} = 0.001$, $p = 0.98$) and between the sexes ($F_{1,20} = 0.26$, $p = 0.62$) with no interactions with treatment (treatment \times sex, $F_{1,20} = 0.08$, $p = 0.79$) or with treatment and post-exposure day (treatment \times post-exposure day \times sex, $F_{1,20} = 1.0$, $p = 0.33$).

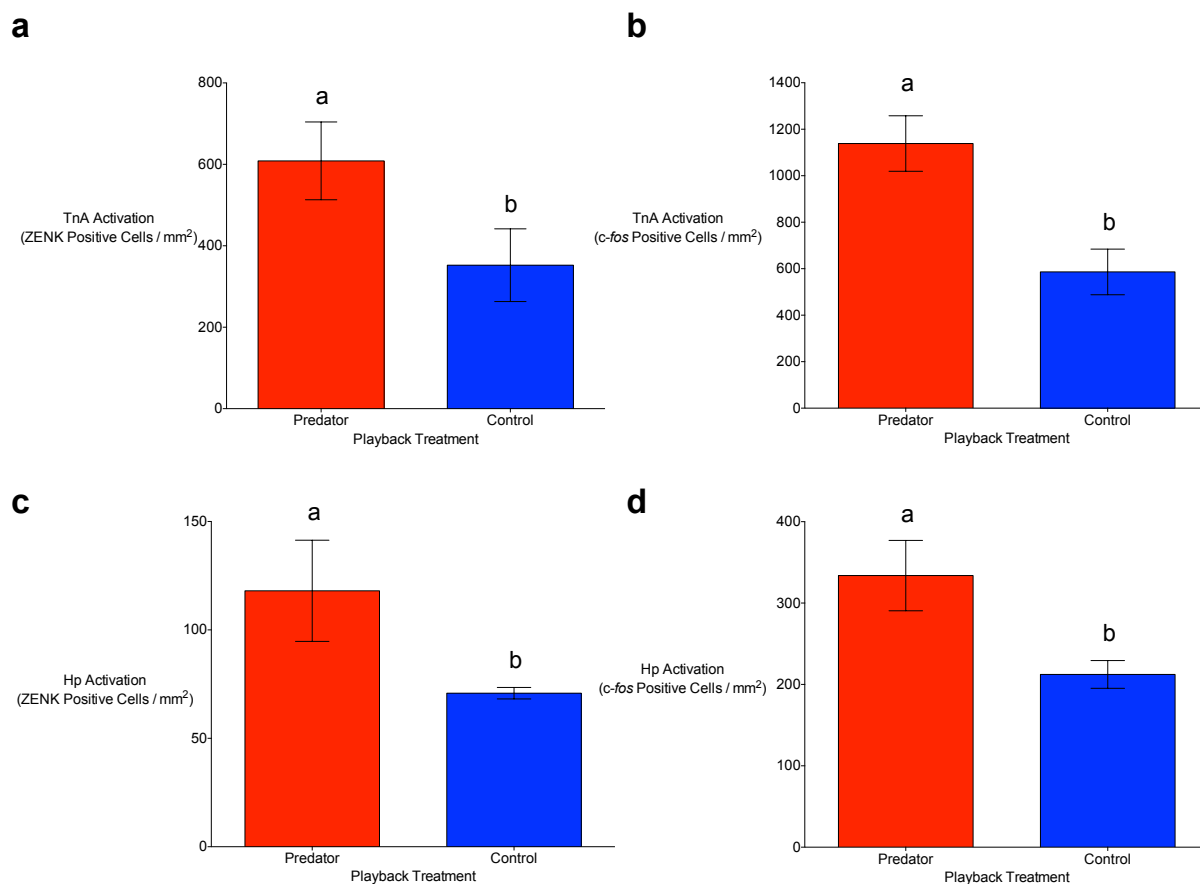


Figure 2.1. Auditory predator playbacks result in significantly higher numbers of ZENK (a) and c-fos (b) positive cells in the nucleus taeniae of the amygdala (TnA) in the brains of black-capped chickadees in comparison to non-predator (control) playbacks. The same effect can be seen in the hippocampus (Hp) of chickadees using ZENK (c) and c-fos (d). Means (\pm SE) represented by different letters are significantly different ($p < 0.05$).

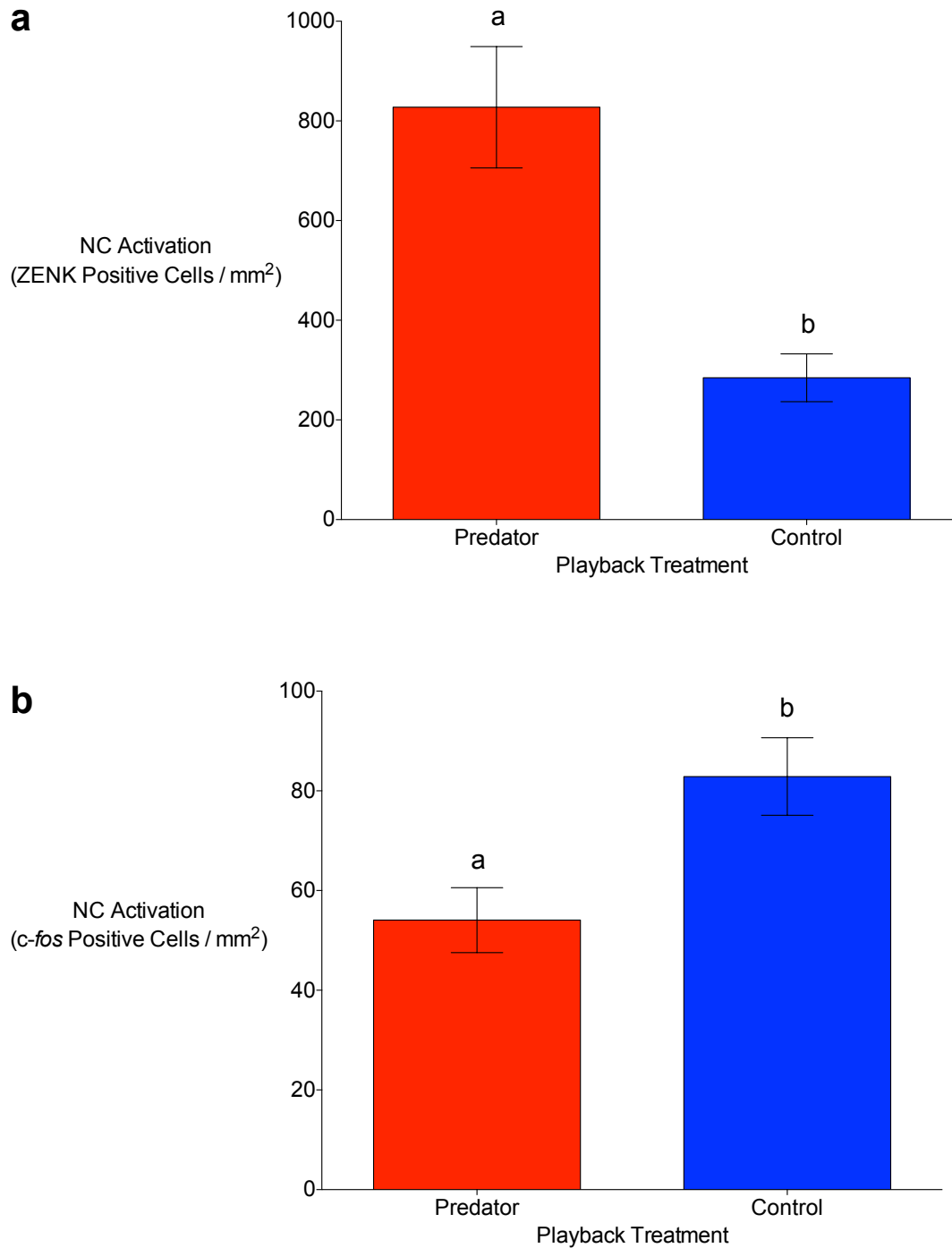


Figure 2.2. The caudal nidopallium (NC) of black-capped chickadees is differentially active in response to auditory predator and non-predator (control) playbacks depending on the immediate-early gene (IEG) used to measure this activation. Using ZENK, the predator treatment resulted in significantly higher NC immunoreactivity than the control treatment (a). The opposite pattern occurred when the IEG *c-fos* was used to quantify the immunoreactivity (b). Means (\pm SE) represented by different letters are significantly different ($p < 0.05$).

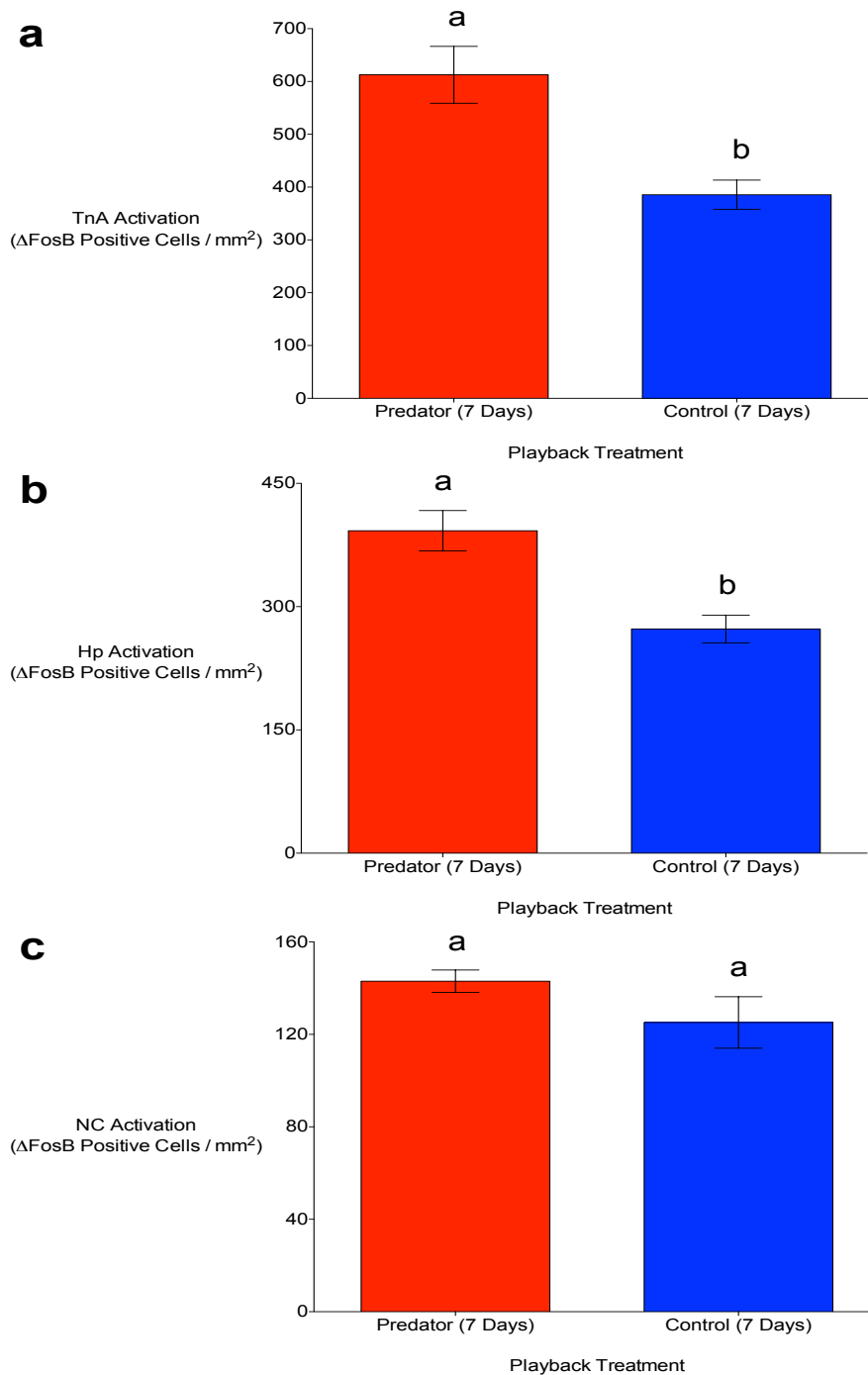


Figure 2.3. In the nucleus taeniae of the amygdala (TnA) (a) and hippocampus (Hp) (b) of black-capped chickadees, there was significantly greater Δ FosB immunoreactivity seven days post-stimulus in response to auditory predator playbacks in comparison to non-predator (control) playbacks. There was no significant difference in Δ FosB immunoreactivity between the two playback treatments in the caudal nidopallium (NC) (c). Means (\pm SE) represented by different letters are significantly different ($p < 0.05$).

2.4 Discussion

I have identified quantifiable immediate and lasting effects of predator-induced fear on three brain regions in wild-caught black-capped chickadees. The TnA, Hp, and NC were active immediately after a brief exposure to a simulated predator, indicating that the activation of these regions is changed by predator exposure. In addition, my results demonstrate lasting effects of a simulation of chronic perceived predation risk on the TnA and Hp for up to a week following re-introduction of wild-caught birds into a semi-natural environment.

My ability to measure effects of fear immediately after and up to a week following predator stimulus exposure in wild animals has considerable implications for the study of ecology and biomedicine. My wild-caught individuals had likely already experienced attempted predation before my experiment, unlike lab-raised, predator-naïve model organisms. This suggests that the lasting neurobiological changes seen in response to my manipulations likely add to pre-existing predator effects in these wild-caught birds, indicating that an increase in predator pressure can have dramatic effects on the neurobiology of wild animals. As well, the changes in brain activation I found in response to realistic predation threat in wild animals may better represent how PTSD in humans results from fear of life-threatening experiences than the laboratory models previously used. The use of a relevant predator stimulus on predator threat-experienced individuals, resulting in significant neurobiological effects, is an improvement upon the current lab mammal model and may allow us to further our understanding of PTSD in humans.

My results provide new insights into the function and activation of three regions of the avian brain—the TnA, Hp, and NC—over the seven days following exposure to a simulation of chronic predation threat. I used the IEG FosB and labelled its protein splice variant Δ FosB, previously used exclusively in mammalian studies, in order to quantify activation in the brains of wild-caught birds for a week after stimulus exposure. This immunohistochemical technique is a novel and useful tool for neuroecologists to employ in future studies of long-term activation in the avian brain.

Predation threat resulted in increased activation in the TnA and Hp for at least a week, as Δ FosB immunoreactivity was elevated in the hours immediately after stimulus exposure as well as for seven days post-exposure to an auditory playback simulation of chronic predation threat. Regardless of which short-term IEG assay was used, the TnA was highly active in response to the threatening calls of a predator. This result, when paired with the TnA activation found at seven days post-exposure, provides strong support for the proposed function of the TnA as a region central to the processing of fear in the avian brain (Cohen & Goff 1978; Charlier *et al.* 2005).

My results complement previous studies finding TnA and mammalian amygdala hyperactivation in response to aversive stimuli (Dielenberg *et al.* 2001; Li *et al.* 2004; Rosen *et al.* 2005; Brito *et al.* 2011; Marzluff *et al.* 2012; Cross *et al.* 2013) by demonstrating that the avian homologue to the mammalian amygdala is activated by auditory cues of predator threat. Elevated Δ FosB immunoreactivity in the TnA for seven days after the predation treatment provides further evidence for the proposed role of the TnA as a region central to a network responding to threatening stimuli in the avian brain (Cohen & Goff 1978). My TnA result mirrors the long-term aberrant medial amygdala

activity characteristic of humans with PTSD in response to both fearful and non-fear related stimuli (Liberzon *et al.* 1999; Protopopescu *et al.* 2005; Rabinak *et al.* 2011), and high amygdala activity seen in humans in response to emotionally aversive stimuli (Cahill *et al.* 1996; McGaugh 2004).

Predator-induced increases in immunoreactivity in the Hp following exposure to the threatening predator treatment for up to a week provide support for its role as the homologue of its mammalian counterpart, and also for its involvement in the formation of fear memories (Colombo & Broadbent 2000; Bingman *et al.* 2003). Increased hippocampal IEG activity is an indication of plasticity in this region and the formation of memory according to mammalian studies (Kim & Diamond 2002; Lam *et al.* 2009), and the heightened Hp activation I saw is an indication that individuals in my study may have been forming memories of the predator fear they experienced. It may also be an indication that my wild-caught birds, which had presumably interacted with predators before capture, were recalling previous predator threats they had experienced and concomitant behavioural changes. Hp activation has been shown to occur in situations where individuals were required to remember a previous learned stimulus (Mayer *et al.* 2010), even in the presence of predator threat (Galliot *et al.* 2010). However, high levels of stress can inhibit Hp-related memory, so the possibility that hippocampal activation is related to the recall of fear memory remains an area for future investigation (Kim & Diamond 2002; Park *et al.* 2008; Wilson *et al.* 2014). Several studies have demonstrated the involvement of the avian hippocampus in the network processing fear in the short-term (Cheng *et al.* 1999; Cross *et al.* 2013). These short-term findings in combination with my long-term results suggest that the hippocampus may be generally involved in the

fear network as a region involved in fear learning and the formation of fear memory. The hippocampus is not merely active during and immediately after a threatening experience, but that the activation of this region continues for at least a week following a threat.

Increased amygdala activity in humans has been found to influence memory processing in other brain regions, like the hippocampus (McGaugh 2000), and activity in these two regions has been found to be correlated in response to emotionally arousing material, likely due to strong bidirectional communication between the two (Dolcos *et al.* 2004; McGaugh 2004; Galliot *et al.* 2010; Chattarji *et al.* 2015). My results may demonstrate a similar pattern of connectivity and activation between the TnA and Hp (Cheng *et al.* 1999; Chattarji *et al.* 2015), as I found that both were significantly activated immediately after exposure and for seven days following exposure to predator cues. Communication and feedback between these two brain regions is an important area for future work.

My findings suggest that an experience of predator threat, or fear, results in lasting changes in the activation of avian brain regions implicated in fear and fear memory, the TnA and the Hp, for at least a week following a simulated predator encounter, complementing previous results that show activation in these areas immediately after predator threat. We may be seeing effects similar to those seen in the human amygdala and hippocampus in the week following a life-threatening experience, and these changes in brain activation may also occur with changes in brain structure and neuroarchitecture, physiology, and behaviour in wild animals, analogous to those we see in humans with PTSD.

The role of the NC in the processing of fear in the avian brain has so far been ambiguously described. NC activation in response to the two treatments in my short-term study differed between the ZENK and *c-fos* assays— increased ZENK activation, but decreased *c-fos* activation in response to the predator treatment when compared to the non-threatening control. In addition, the quantity of *c-fos* positive cells was low. Other studies have shown that different IEGs can demonstrate different sensitivity to the same stimuli, because they are often expressed in a specific population of neurons within a brain region (Sheng & Greenberg 1990; Nastiuk *et al.* 1994; Kimpo & Doupe 1997; Bock *et al.* 2005; Feenders *et al.* 2008). In my long-term study, I found no difference in activation between the predator and non-predator treatments in the NC. Despite findings that this area was active in American crows (*Corvus brachyrhynchos*) immediately following exposure to a predator mount (Cross *et al.* 2013) and my short-term ZENK results, my long-term results suggest several possibilities about the function of the NC.

First, the lack of increased Δ FosB activity in the NC in the week following predator treatment may indicate that neurons in this region do not up-regulate FosB in response to threat, as Δ FosB expression is found in certain subpopulations of neurons, but not all, within regions in the mammalian brain in response to a chronic stimulus (Cunningham *et al.* 2008). This difference in active immediate-early genes is plausible, as the NC and prefrontal cortex are analogous brain regions which differ in location, structure, and innervation, despite having the same proposed function (Rose & Colombo 2005). It is also possible that FosB may be expressed to some degree initially after the threat, but the FosB/ Δ FosB products are broken down in these neurons before I began to label them four days post-stimulus.

In addition, the lack of NC activation in the week following predator threat paired with increased TnA activity may suggest that the NC under normal circumstances inhibits TnA activity, as the prefrontal cortex inhibits the mammalian amygdala (Chattarji *et al.* 2015). Chronic stress in mammals has been shown to weaken structures that provide negative feedback to the stress response, like the mammalian prefrontal cortex, through increases in catecholamines and glucocorticoids. This leads to a shift in function to brain regions promoting the stress response, like the medial amygdala, strengthening their activity (Arnsten 2009). The predator treatment in this study could have resulted in a similar effect in the avian brain, resulting in low NC activation and high TnA activation in response to a simulation of chronic threat, although communication and feedback between these two regions is not yet understood in the avian brain.

As has been found in mammalian and human studies of the effects of chronic stress on the brain, long-term changes in brain activation may indicate changes in synaptic plasticity, and may underlie changes in predator avoidance behaviour, physiology, and brain chemistry (Wiedenmayer 2004); for example, decreased motor activity and increased anxiety (Adamec *et al.* 2004; Mackenzie *et al.* 2010), increased corticosterone and altered neurotransmitter activity (File *et al.* 1993; Wilson *et al.* 2014). These changes are evident in humans with PTSD, who experience persistent poor sleep, anxiety, and hypervigilance, along with increased amygdala, hippocampus, and prefrontal cortex activity for weeks or months (Rabinak *et al.* 2011; Corley *et al.* 2012).

I have begun to identify some lasting neurobiological changes associated with chronic predation stress in the avian brain. Based on results of many human and mammalian studies regarding the parallel lasting physiological and behavioural effects of

chronic stress on the brain, we should expect to see similar changes in wild-caught birds. Many studies have shown that the effects of predator stimuli can be predicted by the intensity of the stimulus a– a dose-response effect (Wiedenmayer 2004). The lasting effects of auditory predator threat on the brain in my study may, then, be smaller than what could be seen if individuals were exposed to a more intense stimulus such as a live predator. It could be expected that behavioural and physiological changes would be more dramatic in response to a more severe stimulus as well.

Further investigation of neurobiological changes resulting from predator-induced fear of differing intensities in wild-caught non-mammals is a critical next step in the study of predator-prey ecology and biomedical fear research. Δ FosB will be a valuable tool for the study of these long-term changes in activation in the avian brain and can be used as a means of investigating the avian brain network processing fear to a greater degree, and to begin to link lasting changes in the brain with effects on behaviour and physiology.

By using wild-caught non-mammals and finding long-term changes resulting from a fearful stimulus proposed to mimic the stimuli resulting in PTSD, I have provided further validation for the biomedical model of fear. By creating a scenario closer to what occurs in the real world, I provide new information to be incorporated into the existing biomedical animal models of human fear-related disorders. The use of wild animals in human biomedical research is novel, but is useful for researchers to better understand the causes and underlying physiological processes leading to human disease. In addition, I expect that my results in combination with future studies of behaviour, physiology and

other neurobiological measurements will provide a more complete picture of the lasting impacts of the fear of predators on wild animals.

Lasting changes in the brain, as seen in humans and other mammals, may occur concomitantly with alterations in physiology and behaviour that persist over time, which may be beneficial if appropriate to the current level of predation risk. However, if these responses persist longer than a threat is present, they may potentially affect an individual's ability to carry out its normal activities, such as foraging or mating. Decreased ability to carry out these vital functions will lead to negative impacts on individual health and survival, potentially affecting the ability to reproduce and effectively raise offspring (Boonstra *et al.* 1998; Zanette *et al.* 2006, 2011; Creel *et al.* 2007; Sheriff *et al.* 2009; Travers *et al.* 2010). Alterations in reproductive success and survival will have impacts at the population, community, and ecosystem level, affecting many more organisms than the predator and prey alone. My study lays the groundwork for the study of lasting neurobiological change in wild animals. Future investigation of these changes and their connections with individual physiology and behaviour up to effects on populations and beyond will provide a more comprehensive view of the severe and long-lasting impacts that predators have on prey survival and demography.

2.5 References

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Chapter 3

3.1 Introduction

The constant and unpredictable threat of predator attack shapes prey ecology, and has broad effects on prey reaching from individual physiology to reproduction and survival (Preisser & Bolnick 2008; Anson *et al.* 2013). Traditional ecological research has viewed predator-prey interactions as solely consumptive, i.e., predators kill prey to eat them, and prey have been viewed as passive victims in this system (Lima 1998; Preisser *et al.* 2005). This view greatly simplifies the roles that both predators and prey play within ecosystems, as the presence of predators affects prey to a far greater degree than by mortality alone (Abrams 1993; Schmitz *et al.* 2004). Predators impact prey not only by how many they kill, but also by how many they threaten (Schmitz *et al.* 2008). As no failure is as serious as the failure to escape a predator— failure to escape invariably results in death— the ability of prey to react to changes in predation risk is fundamental to their survival and future reproductive success (Lima & Steury 2005).

Prey use a variety of anti-predator responses to avoid detection by predators and to escape attack when they experience predator-induced fear (i.e., the prospect of imminent, violent death due to perceived predation risk) (Lima & Dill 1990; Creel & Christianson 2008; Preisser & Bolnick 2008). Increased predation risk can cause prey to intensify anti-predator behaviours in order to become less conspicuous in the presence of predators (Lima 1998; Schmitz *et al.* 2008), for example by altering their habitat use (Creel *et al.* 2005) and foraging (Clinchy *et al.* 2004; Zanette *et al.* 2013). Predator risk and encounters with predators can also impact many physiological measures of stress

(Clinchy *et al.* 2004), like stress hormone levels (Hik *et al.* 2001; Creel *et al.* 2007; Newman *et al.* 2012).

In addition to these individual anti-predator responses, social signals are used by animals to alert conspecifics to the threat of a predator in the environment. These cues can be visual (Hogan & Laskowski 2013), chemical (Sanches *et al.* 2015), or auditory (Hare & Atkins 2001). Black-capped chickadees (*Poecile atricapillus*) use vocalizations to alert flock members to various levels of predation threat, the two most common calls being the *mobbing call* and the *high zee call* (Ficken *et al.* 1978; Smith 1991). These calls, both communicating predator threats of different intensities to conspecifics, are dissimilar in acoustic structure (Ficken *et al.* 1978; Smith 1991).

Chickadee *mobbing calls* are used in the event of moderate predation threat, for instance when one individual in a flock locates a stationary predator. This call warns other chickadees of this potential danger and calls the flock together to mob the predator, chasing it away. The activation in auditory regions (caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM)) of the chickadee brain has been investigated in response to the calls of northern saw-whet owl (*Aegolius acadicus*) predators and chickadee *mobbing calls* (Avey *et al.* 2011). These acoustically different calls resulted in equal activation in the auditory regions, suggesting that these regions are processing the information content of the calls, not their acoustic structure.

The *high zee call* is emitted in instances of high predation threat, for example when a predator is detected by an individual, but has not yet been localized (e.g., an aerial predator in motion). These calls are typically used by male chickadees to alert conspecifics to a threat, resulting in a freezing response from other individuals in the

flock (Smith 1991). Although dissimilar in acoustic structure, both types of chickadee alarm calls result in behavioural changes within the flock that contribute to predator avoidance. It can be expected, then, that these calls may result in similar individual neurobiological responses as the calls of predators themselves.

Little is known about how avian brain regions are activated in fear processing, or how fear is processed in the brains of wild animals. My study used auditory playbacks of social cues of predation threat to examine activation in the TnA, Hp, and NC in wild-caught black-capped chickadees (*Poecile atricapillus*) using two immediate-early gene (IEG) assays. I used auditory playbacks of black-capped chickadee *high zee calls*, chickadee *mobbing calls*, and red-breasted nuthatch calls to simulate high predation threat, moderate predation threat, and no threat, respectively. I then quantified activation in response to these playbacks in the TnA, Hp, and NC of each individual by labelling the protein products of the IEGs ZENK and *c-fos* in active nuclei.

I predicted that the amount of activation seen in each brain region would be proportional to the level of threat encoded in each call. I also expected to see some differences between the two IEG assays, as differences between the expression of ZENK and *c-fos* have previously been found in different populations of neurons within the same brain region (Sheng & Greenberg 1990; Nastiuk *et al.* 1994; Kimpo & Doupe 1997; Bock *et al.* 2005). I report that the TnA was significantly activated in response to the highest level of threat in both assays, the Hp was activated in response to any threat in both assays, and that the NC activated strongly to threat in the ZENK assay only.

3.2 Methods

Overview

I used wild-caught black-capped chickadees to investigate the effects of three different levels of perceived predation risk in three regions of the avian brain and manipulated this perceived predation risk using auditory playbacks. I used two black-capped chickadee social alarm calls representing different levels of perceived predation threat: *mobbing calls* to simulate moderate-intensity predation threat, and *high zee calls* to simulate high-intensity predation threat. The non-threatening calls of red-breasted nuthatches (*Sitta canadensis*) acted as my control. I used two immediate-early gene assays (ZENK and *c-fos*) to quantify immunoreactivity, and thus activity, in brain regions thought to be involved in processing predator risk: the nucleus taeniae of the amygdala (TnA), the hippocampus (Hp), and the caudal nidopallium (NC).

Perceived Predation Risk Manipulation

Between November 2012 and February 2013, I captured 19 black-capped chickadees (8 M; 11 F, all after hatch year) using seed-baited Potter traps and mist nets from several sites at the University of Western Ontario in London, Ontario, Canada (43°00'37" N, 81°16'47" W). I weighed each individual and sexed all birds based on wing chord length. I housed chickadees in groups of three to eight in semi-natural outdoor aviaries for at least seven days following capture and before manipulations began to acclimate to captivity. Chickadees had access to Mazuri small bird diet PMI Nutrition International, LLC, Brentwood, MO, black oil sunflower seeds, mealworms, and water *ad libitum*.

I randomly assigned six chickadees (seven in the *high zee* group) to each of the

three playback treatments (control: three M, three F; *mobbing call*: four F, two M; *high zee call*: four F, three M). Then, I relocated chickadees to individual cages (25 cm × 30 cm × 37 cm), each placed within an individual sound-attenuating acoustic chamber (Industrial Acoustics Company, Inc., Bronx, NY) for 24 h before the treatment began. Each chamber was set up as described in Chapter 2. I obtained red-breasted nuthatch playbacks from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA). I obtained chickadee *mobbing* and *high zee* alarm calls by exposing wild-caught black-capped chickadees from the University of Western Ontario population to a taxidermic mount of northern saw-whet owls. I organized vocalizations for each treatment into playlists consisting of three calls, each from a different individual (i.e. individuals a-b-c; d-e-f etc.), with each call playing for 5 s, followed by 45 s of silence. This 60 s playlist repeated 30 times at 74 dB SPL (following Avey *et al.* 2011) (Appendix B). At this time, I turned off the chamber lights and individuals remained in the silent, dark auditory chamber for 90 min. Euthanasia and perfusion was carried out as described in Chapter 2.

Brain Processing

I carried out brain slicing and immunohistochemistry for ZENK and *c-fos* as described in Chapter 2. I quantified immunoreactivity in the nucleus taeniae of the amygdala (TnA), hippocampus (Hp), and caudal nidopallium (NC) for each slice with the region of interest identifiable in both hemispheres (Appendix A). Image capture and cell counting are described in Chapter 2. I captured all images and counted all cells without knowing which treatment each slice belonged to, so as not to bias the results.

Statistical Analyses

I calculated mean counts of IEG positive cells / mm² within each brain region in each individual, and then calculated a mean per treatment within each brain region. I then compared the mean count of IEG positive cells / mm² for each treatment group within each brain region using 2-factor ANOVAs with treatment and sex as fixed factors, followed by Dunnett's post-hoc test comparing the *mobbing call* and *high zee* treatments with the non-threatening control. Sex was included as a fixed factor in all statistical analyses in order to rule out any possible sex effects, as most mammalian research is done exclusively on males.

Prior to parametric analyses with SPSS Statistics for Macintosh (Version 20.0; IBM Corporation, Armonk, NY, USA, 2011), all data were Box-Cox transformed and checked for normality of error and homogeneity of variances. I present untransformed means \pm SE for clarity.

3.3 Results

The TnA, the proposed centre of the avian fear network, exhibited its highest level of activation in response to the high perceived predation risk (chickadee high zee call) treatment, for both ZENK and *c-fos* (Figs. 3.1.a,b; two-factor ANOVA with sex as a fixed factor: treatment, $F_{2,12} = 6.6$, $p = 0.01$; $F_{2,11} = 4.3$, $p = 0.04$, respectively). The high-risk treatment resulted in significantly greater immediate-early gene expression than the control treatment (Dunnett's test comparing control treatment to high zee: ZENK, $p = 0.01$; *c-fos*, $p = 0.02$). Chickadee *mobbing calls* signalling moderate intensity predation risk, however, generated relatively little immediate-early gene expression in the TnA, and was not significantly different from that generated as a result of the control treatment for

both ZENK (Dunnett's test comparing control treatment to mobbing: $p = 0.6$) and *c-fos* (Dunnett's test comparing control treatment to mobbing: $p = 0.6$). I found no significant main effects of sex with either assay (sex, ZENK: $F_{1,12} = 0.2$, $p = 0.66$; *c-fos*: $F_{1,11} = 0.02$, $p = 0.89$), and both sexes responded similarly across the three treatments for the ZENK (treatment \times sex: $F_{2,12} = 2.9$, $p = 0.10$) and *c-fos* assays (treatment \times sex: $F_{2,11} = 1.8$, $p = 0.35$).

The hippocampus, involved in spatial processing and the formation of fearful memories, was significantly more active than the controls in response to the moderate-risk and high-risk treatments for both ZENK (Fig. 3.2.a; two-factor ANOVA with sex as a fixed factor: treatment, $F_{2,13} = 23.9$, $p < 0.001$) and *c-fos* (Fig. 3.2.b; two-factor ANOVA with sex as a fixed factor: treatment, $F_{2,11} = 13.5$, $p = 0.001$). Expression was significantly higher after exposure to high intensity (Dunnett's testing control vs. high zee calls: ZENK, $p < 0.001$; *c-fos*, $p < 0.001$) and moderate intensity (Dunnett's testing control vs. mobbing calls: ZENK, $p < 0.001$; *c-fos*, $p = 0.01$) predation risk calls. These effects were consistent between the sexes for ZENK (treatment \times sex, $F_{2,13} = 0.40$, $p = 0.68$; sex, $F_{1,13} = 1.7$, $p = 0.22$) and *c-fos* (treatment \times sex, $F_{2,11} = 0.52$, $p = 0.61$; sex, $F_{1,11} = 0.39$, $p = 0.55$).

The caudal nidopallium (NC), proposed analogue to the mammalian prefrontal cortex, showed differential activation among the three playback treatments for ZENK (Fig. 3.3.a; two-factor ANOVA with sex as a fixed factor: treatment, $F_{2,11} = 17.3$, $p < 0.001$) and no difference among the three treatments for *c-fos* (Fig. 3.3.b; two-factor ANOVA with sex as a fixed factor: treatment, $F_{2,11} = 0.83$, $p = 0.46$). I found significant differences in ZENK expression between the control treatment and both the high and

moderate risk calls (Dunnett's test: $p < 0.001$ and $p = 0.001$, respectively), and these effects remained consistent between the sexes (treatment \times sex: $F_{2,11} = 0.6$, $p = 0.59$; sex: $F_{1,11} = 1.7$, $p = 0.22$). Conversely, *c-fos* expression in the NC was not significantly different between the control treatment and high-risk calls (Dunnett's test, $p = 0.96$) and the control treatment and moderate risk calls (Dunnett's test, $p = 0.86$). Females and males had similar *c-fos* expression in the NC (sex, $F_{1,11} = 3.5$, $p = 0.09$), and no treatment by sex interaction was found (treatment \times sex, $F_{2,11} = 1.6$, $p = 0.25$).

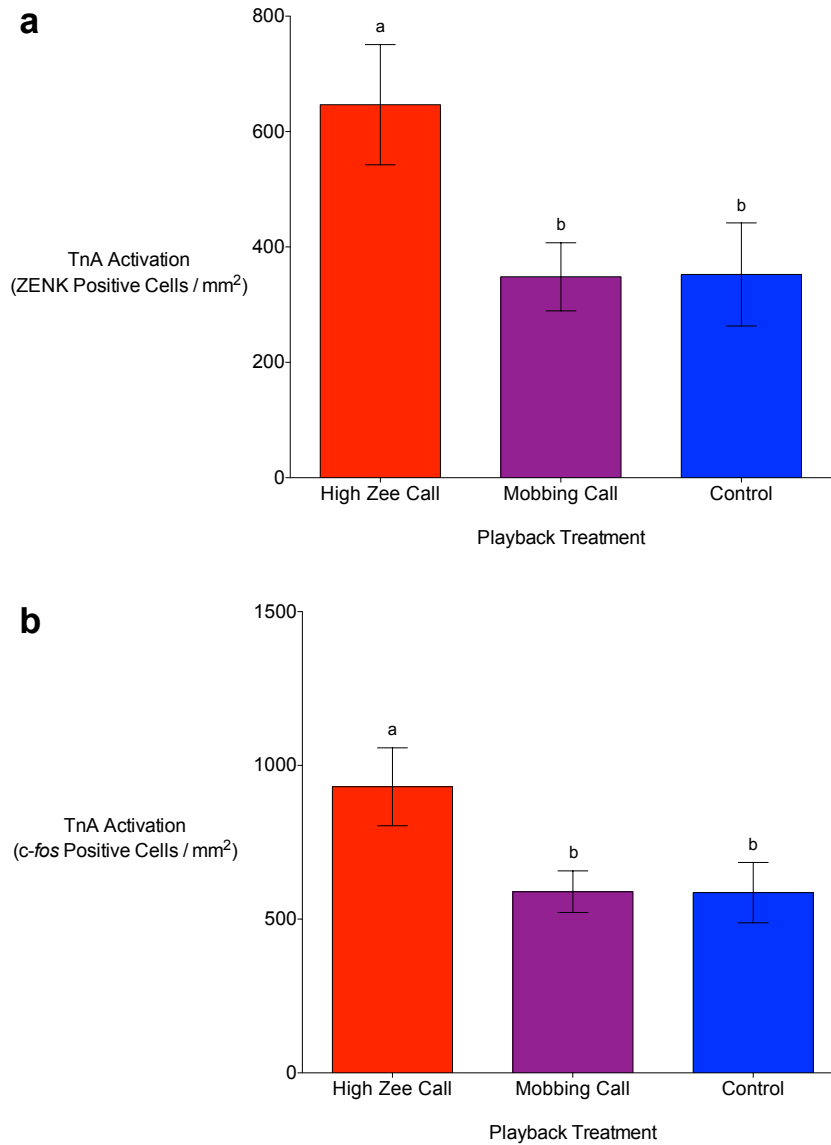


Figure 3.1. Auditory playbacks of chickadee *high zee calls* result in significantly higher numbers ZENK (a) and *c-fos* (b) positive cells in the nucleus taeniae of the amygdala (TnA) in the brains of black-capped chickadees in comparison to auditory chickadee *mobbing call* and non-predator (control) playbacks. Means (\pm SE) represented by different letters are significantly different from the control ($p < 0.05$).

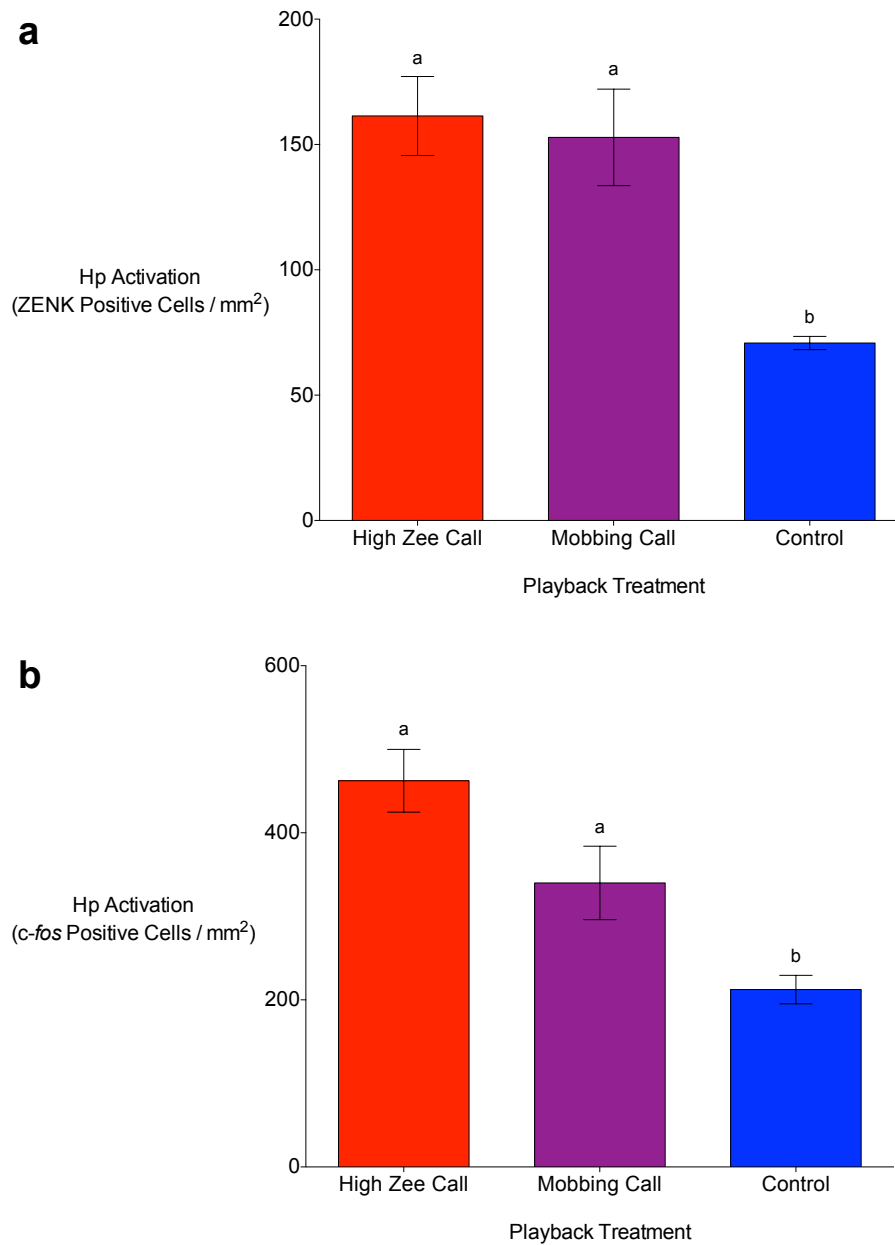


Figure 3.2. Auditory playbacks of chickadee *high zee* and *mobbing calls* result in significantly higher numbers ZENK (a) and *c-fos* (b) positive cells in the hippocampus (Hp) in the brains of black-capped chickadees in comparison to non-predator (control) playbacks. Means (\pm SE) represented by different letters are significantly different from the control ($p < 0.05$).

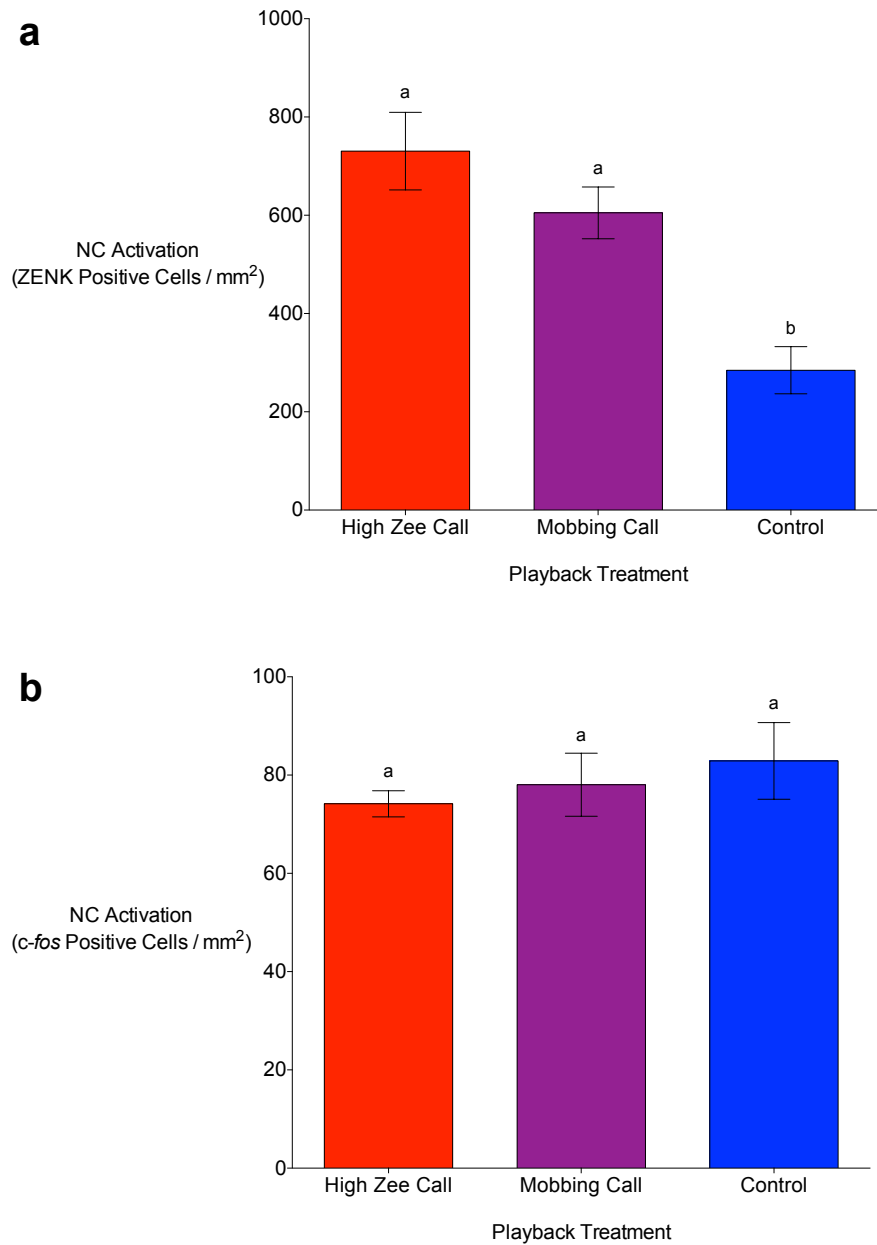


Figure 3.3. Auditory playbacks of chickadee *high zee* and *mobbing calls* result in significantly higher numbers ZENK (a) positive cells in the caudal nidopallium (NC) in the brains of black-capped chickadees in comparison to non-predator (control) playbacks. Chickadee *high zee* and *mobbing call* playbacks do not result in significantly different *c-fos* NC activation from the controls (b). Means (\pm SE) represented by different letters are significantly different from the control ($p < 0.05$).

3.4 Discussion

I have identified quantifiable differences between the processing of two conspecific predator alarm calls, each indicating a different level of predator threat, in three regions of the wild-caught black-capped chickadee brain. Using two different IEG assays, the TnA was highly active when compared to the control in response to the chickadee *high zee call*, a social signal of extreme alarm, but not in response to the chickadee *mobbing call*, a signal of moderate alarm. I found that the Hp was significantly activated in response to both chickadee alarm calls, regardless of which assay I used to quantify this activation. The NC exhibited the same activation as the Hp—equally increased activity in response to both alarm calls—when I used the ZENK assay, however the *c-fos* assay revealed no differences in activation from the control for either alarm call.

These results indicate that the brains of wild, social animals do not process predator-induced fear only by discriminating between predator and non-predator stimuli. In fact, I demonstrated that conspecific alarm calls signifying different levels of predation threat result in significant activation in brain regions thought to be involved in avian fear processing. Most strikingly, I found that in one of these regions, activation depends on the level of threat encoded within the call, not the type of call itself. A similar result was previously found in the auditory regions of the black-capped chickadee brain (the caudomedial mesopallium, CMM, and caudomedial nidopallium, NCM) (Avey *et al.* 2011). These brain regions showed no significant difference in ZENK expression when chickadees were exposed to conspecific *mobbing calls* or northern saw-whet owl calls. Although these calls are acoustically different, the brain was equally immunoreactive, suggesting that the information content of the calls—signifying predator threat—and not

their acoustic characteristics, resulted in similar brain activation (Avey *et al.* 2011).

These results, combined with results from my study, have profound implications for the study of avian neurobiology, and suggest that the proposed avian fear network may be more sophisticated than previously thought.

I found that the TnA, proposed centre of the avian fear network, attends differently to social alarm calls that encode different levels of threat. My results show that only the chickadee *high zee* call results in a significant increase in TnA activation from the control in wild-caught black-capped chickadees, with no significant increase in activation in response to the chickadee *mobbing call*. *High zee calls* indicate extreme predation threat, while *mobbing calls* indicate only a moderate level of predation threat. It appears that the TnA processes these calls depending on perceived threat level. Not only is the TnA active to aversive stimuli generally (Brito *et al.* 2011) and visual or auditory predator cues (Marzluff *et al.* 2012; Cross *et al.* 2013; Chapter 2), but results of the current study show that this area may be involved in processing more ambiguous social cues of high predator threat, generally attending to only the most threatening cues. This further reinforces the proposed role of this region as central to the network processing fear in the avian brain (Cohen & Goff 1978; Marzluff *et al.* 2012).

The Hp exhibited significantly increased activation in comparison to the control in response to both the high- and moderate-threat conspecific alarm calls in both assays. This indicates that although this area is attending to both social cues of predator threat, it does not have the same level of specificity as the TnA, and does not attend differently to moderate versus high threat cues. Where the TnA appears to be attending to the *level* of threat encoded in conspecific calls with its activation increased only in response to high

threat, the Hp appears only to differentiate between cues of predator threat and non-threatening cues. This result aligns with previous investigations of the Hp and its involvement in the avian fear network in the processing and formation of memory (Clayton & Lee 1998; Mayer *et al.* 2010; Nishizawa *et al.* 2011; Cornwell *et al.* 2012), as well as in the processing of predator cues specifically (Cross *et al.* 2013). This also supports the results of my previous study, which showed increased Hp activation in response to predator cues in both the short- and long-term (Chapter 2). Overall, it appears that the Hp is involved in the processing of predator threat, in the form of both auditory predator calls and social cues of predators.

In the ZENK assay, the NC exhibited a similar pattern to the Hp, and was significantly active in comparison to the control in response to both threatening treatments. However, in the *c-fos* assay, no differences were found among the three treatments, and the quantity of *c-fos* positive cells was low. Other studies have shown that different IEGs can demonstrate different sensitivity to the same stimuli, because they are often expressed in a specific population of neurons within a brain region (Sheng & Greenberg 1990; Nastiuk *et al.* 1994; Kimpo & Doupe 1997; Bock *et al.* 2005; Feenders *et al.* 2008). I found differences in ZENK and *c-fos* in the NC in my previous study comparing predator and non-predator calls, in which ZENK activation increased in the predator treatment and *c-fos* activation decreased (Chapter 2). These results taken together indicate that this brain region may contain two populations of neurons, one expressing ZENK and one expressing *c-fos*, which are activated in response to different stimuli.

When I quantified ZENK expression in the NC I found significant activation to each of the chickadee alarm calls. This is consistent with the proposed role of this region in fear processing and decision-making (Rose & Colombo 2005; Cross *et al.* 2013), as individuals confronted with a fear-inducing stimulus should have active decision-making processes. This result is consistent with the significantly increased ZENK expression in the NC in response to predator calls that I found in a previous study (Chapter 2). However, in the same study I found decreased *c-fos* expression and no change in Δ FosB expression in the NC in response to predator calls (Chapter 2). Taken together, these results indicate that the role of the NC in fear processing continues to be somewhat ambiguous and that further investigation of the function of the NC and IEG activity present in NC neurons is required.

Overall, my results indicate that the processing of predator-induced fear in wild animals is not solely a matter of differentiating between predator and non-predator cues—social cues of predator threat are attended to as well. The fact that we can measure fear in response to social cues of threat, along with direct predator stimuli, expands our knowledge of neuroecology and fear processing in the avian brain specifically. In order to better understand the effects of activation in the TnA, Hp, and NC, analysis of behaviours resulting from exposure to conspecific alarm calls is crucial. Investigation of physiological changes and behaviours occurring concomitantly with altered brain activation is the next step in the study of the neurobiology of predator-prey interactions and will result in a more holistic understanding of the impacts of predator-induced fear on their prey. Altered physiology and behaviour as a result of perceived predation risk may negatively impact individual foraging and habitat use, which in turn may impact

reproduction, parenting and survival (Clinchy *et al.* 2013). Taken together, my results demonstrate the ability of three regions of the wild-caught chickadee brain to process and differentially activate in response to social cues of predation threat, providing further insight into the functions of these regions and laying the groundwork for further studies of neuroecology.

3.5 References

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Chapter 4

General Discussion

The goals of this thesis were to explore the effects of life-threatening predator fear (i.e., the prospect of imminent, violent death due to perceived predation risk) on the brains of wild-caught animals, to investigate the functions of several avian brain regions, to expand on the biomedical model of fear central to our understanding of post-traumatic stress disorder (PTSD) in humans, and to further our knowledge of predator-prey ecology. In Chapter 1, I reviewed how fear, including the fear of predators, has acute and lasting impacts on organisms. I provided a specific focus on the neurobiological impacts of life-threatening fear, our understanding of which comes mostly from studies of PTSD, and identified some mammalian brain regions affected by this fear. Finally, I identified a gap in our knowledge surrounding the impacts of predator-induced fear on the brains of wild animals, and specifically on birds. I introduced three brain regions of interest thought to play a role in fear processing in the avian brain. In Chapter 2, I explored the short- and long-term effects of predator-induced fear on the nucleus taeniae of the amygdala (TnA), hippocampus (Hp), and caudal nidopallium (NC) in the brains of wild-caught black-capped chickadees (*Poecile atricapillus*). I manipulated perceived predation risk using auditory playbacks of predator calls and used two short-term immediate-early genes (IEGs) (ZENK and *c-fos*) and one long-term IEG (FosB) to quantify activation in the TnA, Hp, and NC. These studies allowed me to provide support for the proposed roles of these brain regions in avian fear processing, and to demonstrate that the fear of predators has lasting effects on the brains of wild animals. In Chapter 3, I investigated whether these same three avian brain regions were activated by conspecific social cues of

threat. I used black-capped chickadee *mobbing* and *high zee* calls to simulate moderate and high predation risk, and quantified ZENK and *c-fos* immunoreactivity. The results of this study indicate that fear processing in the avian brain is more sophisticated than previously thought, in that social cues of threat are processed, and even discriminated between, in the TnA, Hp, and NC. In this final chapter, I aim to summarize the broader significance of my findings to the fields of avian neurobiology, biomedical research, and applied conservation. I also outline some future directions for the fields of neuroecology, biomedicine, and predator-prey ecology in general.

4.1 Perceived predation threat results in short-term and lasting changes in the avian brain

I tested how predation threat affected activation of the TnA, Hp, and NC of wild caught birds over the short- and long-term. My first study (Chapter 2) sought to determine whether these three brain regions were activated by predation threat in the short-term, and to lend support for the proposed roles of these regions in avian fear processing. The TnA and Hp were both significantly more active in response to the calls of a predator compared to a non-threatening control when both ZENK and *c-fos* immunoreactivity were quantified. The NC was significantly more active when ZENK was quantified as well, but not when *c-fos* was quantified. This study demonstrated that the TnA and Hp are likely involved in fear processing in the avian brain, and that the NC may also be involved.

My long-term study using the IEG FosB allowed me to further clarify the results of my short-term study. Results from my investigation of long-term brain activation in response to predator calls showed that both the TnA and Hp remained significantly active

for at least a week following exposure to auditory predator playbacks, demonstrating a lasting impact of predator threat on the brain. The NC, on the other hand, was not more active than in response to the control one week after predator threat, indicating that this region may not express Δ FosB or may not be affected by predation threat over the long-term.

In addition to testing the effect of predator calls on the TnA, Hp, and NC, I used playbacks of chickadee *mobbing* and *high zee* calls in order to examine these same brain regions in response to social cues of moderate and high predation threat. The TnA was more active in response to *high zee calls* in comparison to the *mobbing* and control calls, indicating that this region not only processes predator cues themselves, but also to conspecific cues of high predation threat. Interestingly, this region also appears to discriminate between different conspecific cues of threat, attending significantly more to the more threatening conspecific cue, the *high zee call*. The Hp was active in response to both conspecific alarm calls, showing that this region processes social and direct cues of predation threat. Finally, the NC again was active in response to both alarm calls, but only when quantified using the ZENK assay.

In all, my results help to clarify the roles of the TnA, Hp, and NC in fear processing in the avian brain. The TnA is active in the short- and long-term in response to calls of predators themselves, and also in response to a social cue of high predation threat. This lends support to the proposed role of this region as central to the avian fear network (Cohen & Goff 1978; Charlier *et al.* 2005; Marzluff *et al.* 2012), and also mirrors results about its homologue, the mammalian medial amygdala, which has been found to be active in response to fearful stimuli, including predator cues (Dielenberg *et*

al. 2001; Li *et al.* 2004; Rosen *et al.* 2005; Shin *et al.* 2006; Shiromani *et al.* 2009; Gross & Canteras 2012; Chattarji *et al.* 2015). The Hp was also active in the short- and long-term in response to the calls of predators themselves and to both conspecific alarm calls. These results again corroborate the proposed role of this region (Colombo & Broadbent 2000; Nishizawa *et al.* 2011; Cross *et al.* 2013), and also align with results from human and mammalian studies, showing activation in response to threatening stimuli (Kim & Diamond 2002; Shiromani *et al.* 2009; Cohen *et al.* 2012; Gross & Canteras 2012; Wang *et al.* 2013; Chattarji *et al.* 2015). Finally, the NC was active in response to predator cues and social cues of threat when ZENK immunoreactivity was quantified, but not when *c-fos* was quantified. Additionally, no long-term activation was seen in this region. My ZENK results support the proposed role of this region in fear-related decision-making (Rose & Colombo 2005; Cross *et al.* 2013) and mirror increased activity in its mammalian homologue in response to aversive stimuli (Shin *et al.* 2006; Akirav & Maroun 2007; Shiromani *et al.* 2009; Cohen *et al.* 2012; Gross & Canteras 2012; Maroun 2012; Chattarji *et al.* 2015). My *c-fos* and Δ FosB results, however, have not clarified the function of the NC overall.

It is evident from my results that the threat of predation has quantifiable, lasting impacts on activation in the brains of wild animals, and that these neurobiological changes may have yet unknown impacts on other aspects of individual biology. I have also demonstrated that the avian fear network is sophisticated and shows discrimination between different cues of predator threat. It is evident that the neurobiological impacts of predators on living prey must be taken into consideration in the study of predator-prey ecology and conservation. Additionally, information about the lasting impacts of fear on

the brains of wild animals must be incorporated into the biomedical model of fear in order to better understand the impacts of fear in all organisms, including humans.

4.2 Consequences for avian neurobiology

Results of my studies support the idea that the TnA (Cohen & Goff 1978; Charlier *et al.* 2005; Marzluff *et al.* 2012) and Hp (Colombo & Broadbent 2000; Nishizawa *et al.* 2011; Cross *et al.* 2013) are involved in fear processing in the avian brain, as components of the avian fear network. The activation of these regions over the week following exposure to predator calls as well as their activation in response to social cues of predation threat furthers our understanding of where these threatening cues are processed in the avian brain. In addition, the fact that the TnA is significantly active in response to predator calls and chickadee *high zee calls*, but not in response to chickadee *mobbing calls* suggests that this region is more sophisticated than previously known, in that it processes social cues signifying different degrees of threat differently.

Despite its proposed role as the analogue of the mammalian prefrontal cortex (Rose & Colombo 2005), our NC results did not unambiguously support this. When I quantified ZENK activation in this region, it was active in response to predator calls and both chickadee alarm calls. However, my *c-fos* and Δ FosB assays resulted in no differences between threat and control treatments. Although my ZENK results indicate that this region may be involved in fear processing, further investigation is required in order to determine why my *c-fos* and Δ FosB assays did not reveal similar patterns of activation. Overall, my studies have provided some insight into the functions of the TnA, Hp, and NC in the avian brain to be incorporated into future studies of avian neurobiology.

Lastly, my successful FosB protocol provides a novel tool for avian neurobiological research. The ability to quantify Δ FosB expression provides a new way to examine long-term activity (McClung *et al.* 2004; Nestler 2008) in the avian brain, which was previously done with the use of expensive scans (e.g. PET, fMRI) which have limited availability (Marzluff *et al.* 2012; Cross *et al.* 2013). The FosB technique will prove useful in future studies of long-term activation in the avian brain in response to a variety of stimuli.

4.3 Consequences for biomedical fear research

From studies of humans with PTSD and mammalian laboratory models of this disorder, it is clear that the mammalian brain is changed by fear (Adamec & Shallow 1993; Adamec *et al.* 2004; Shiromani *et al.* 2009; Clinchy *et al.* 2010; Cohen *et al.* 2012; Wotjak & Pape 2013; Chattarji *et al.* 2015). Human and mammalian model studies are the foundation of our knowledge about human fear-related disorders and are the basis of our understanding of the aetiology and treatment of these disorders. However, levels of behavioural and physiological dysfunction similar to those seen in PTSD patients have occurred in wild animals exposed to predator fear (Kavaliers & Choleris 2001; Wiedenmayer 2004; Creel & Christianson 2008; Hawlena & Schmitz 2010; Zanette *et al.* 2011), indicating that wild animals exposed to relevant, seemingly life-threatening predator cues may be more advantageous for the study of fear than previous biomedical models (Wiedenmayer 2004; Clinchy *et al.* 2010, 2013; Cohen *et al.* 2012). The lasting neurobiological effects of predator-induced fear on wild animals that I found in my study may be found to mimic the symptoms of PTSD patients to an even greater degree than those already demonstrated in the lab, as this perceived predation risk is life-long and the

consequence of attack is death (Creel & Christianson 2008; Clinchy *et al.* 2010, 2013; Boonstra 2013). The fact that I could quantify lasting impacts of a relevant fear-inducing stimulus on the brains of wild animals suggests that the use of wild animals and predator threat may become a standard model for further investigating PTSD and other fear-related disorders (Cohen *et al.* 2012). This wild animal model likely better mimics the causes and symptoms of PTSD better than any model currently used in the laboratory, and may lead to improvements in treatment for the approximate 8% of the population who suffer from PTSD (Shiromani *et al.* 2009).

4.4 Consequences for applied conservation

Recent studies have emphasized that the indirect effects of perceived predation risk are ubiquitous factors shaping ecology, from effects on individuals to effects on entire communities (Preisser *et al.* 2005; Creel & Christianson 2008; Sih *et al.* 2010; Macleod *et al.* 2014). In conservation and management, the focus is often on the direct effects of predators, and how to protect prey from these predator attacks (Johnson & Oring 2002; Isaksson *et al.* 2007). This is an important goal as predators, especially those introduced into ecosystems, have direct negative impacts on mammalian and avian prey populations (Salo *et al.* 2007; Anson *et al.* 2013). However, concentrating on protecting prey from attack by their predators at the expense of neglecting to consider the indirect effects of predators is problematic, as the indirect effects of predators have been shown to have broad and dramatic effects on the success of their prey across taxa (Krebs *et al.* 1995; Boonstra *et al.* 1998; Creel *et al.* 2005, 2007; Sheriff *et al.* 2009). Focusing only on direct predation greatly underestimates the impacts that predators have on ecosystems (Schmitz *et al.* 2014; Peckarsky *et al.* 2015). In fact, by eliminating direct predation,

several studies have demonstrated large reductions in prey success due solely to the indirect effects of predators. For example, exposing grasshoppers (*Melanoplus femurrubrum*) to spiders (*Pisurina mira*) with their mouthparts glued shut so they could only threaten their prey resulted in a 29% decrease in a grasshopper population (Schmitz *et al.* 1997). Using netting to protect nestlings from direct predation while broadcasting predator calls to simulate predation risk resulted in a 40% reduction in offspring production by song sparrows (*Melospiza melodia*), despite the fact that no predator attacks occurred (Zanette *et al.* 2011). It is estimated that by ignoring the indirect effects of perceived predation risk, many impacts of predators on their prey may be missed (Luttbeg & Kerby 2005; Preisser *et al.* 2005). My results demonstrate some neurobiological effects that predators have on wild animals. When considered along with the physiological (Hik *et al.* 2001; Apfelbach *et al.* 2005; Slos & Stoks 2008; Sheriff *et al.* 2009; Hawlena & Schmitz 2010) and behavioural effects (Abrams 1986, 1993; Lima & Dill 1990; Brown & Kotler 2004; Creel *et al.* 2005) of predators already found, these results suggest that an environment of high predation risk is unfavourable to prey population success. Increased predation risk may be detrimental to individual foraging (Brown & Kotler 2004; Heithaus & Dill 2006; Zanette *et al.* 2013), result in altered habitat use (Creel *et al.* 2005; Eggers *et al.* 2006), and cause declines in reproductive success (Eggers *et al.* 2006; Zanette *et al.* 2006, 2011; Creel *et al.* 2007; Travers *et al.* 2010). Consequently, these effects can prevent the continued success of a prey population (Luttbeg & Kerby 2005; Preisser *et al.* 2005). Declines in prey populations can, in turn, have dramatic consequences for entire ecosystems (Schmitz *et al.* 2004, 2014; Estes *et al.* 2011). Accordingly, the common management practice of protecting prey from direct

predation while allowing predators to remain in the area may not be sufficient to prevent declines in the populations of prey. Limiting predator presence in ecosystems where prey species are struggling may then be the most effective means by which to allow prey populations to succeed, for example by the removal of introduced predators. Although potentially undesirable to the public, predator removal programs may be the only effective way to allow threatened prey populations to rebound.

My results, while not directly measuring prey success or survival, provide a picture of some of the indirect effects predators have on their prey. When combined with existing knowledge of detrimental behavioural and physiological effects of predation, it is clear that the potential neurobiological effects of predators need to be considered by conservation managers if programs to assist in the recovery of prey populations are to be successful. Going forward, managers must consider the obvious direct impacts of predators on their prey with the less apparent, yet no less important, indirect effects of predators in order to have a full understanding of the impacts of predators within ecosystems and to implement successful population management.

4.5 Future directions

The study of the indirect effects of predators on their prey is relatively new, however it is apparent that these effects are likely to occur in all ecosystems. The study of the neurobiological impacts of predators on their prey is especially novel, and thus there is still much to be determined. My results indicate that we can quantify the fear of predators in the brains of wild prey using auditory playbacks simulating different degrees of predation threat. My studies have begun to clarify the roles of three avian brain regions

in the processing of predator fear, and have laid the groundwork for the further study of the impacts of predators on the brains of their prey.

My results corroborate the proposed functions of the TnA (Cohen & Goff 1978; Charlier *et al.* 2005; Marzluff *et al.* 2012) and Hp (Colombo & Broadbent 2000; Nishizawa *et al.* 2011; Cross *et al.* 2013) of birds in the processing of predator-induced fear. By demonstrating that these regions are active in response to predation threat both immediately post-stimulus and a week after exposure, I suggest that further investigation into the long-term function of these regions is required. As my NC results did not provide the same level of support for the proposed role of this region in fear processing (Rose & Colombo 2005), further investigation into the function and composition of this region is required. The makeup of this region, as well as that of the TnA and Hp, with regards to subpopulations of neurons present, should be investigated in order to determine why my results using different IEGs were not consistent, as has been seen in other studies using multiple IEGs (Sheng & Greenberg 1990; Nastiuk *et al.* 1994; Kimpo & Doupe 1997; Bock *et al.* 2005). In addition, innervation and communication among these three regions must be studied. Increased knowledge of the connections and related activity among these regions will potentially allow future research to determine if this avian fear network functions similarly to the mammalian fear network, where innervation and communication is already well understood (Gross & Canteras 2012; Chattarji *et al.* 2015). Taken together, a more complete study of the function and composition of the TnA, Hp, and NC individually, as well as an investigation into their function as a network, will result in an increased understanding of the avian brain, as well as useful information to be incorporated into the biomedical model of fear.

The use of non-invasive brain imaging techniques (e.g. PET scans) is a logical next step in the study of predator-induced fear and its long-term impacts on the brain. These techniques have been used successfully on captive animals; for example, PET scans have been used on avian species to analyse brain activity (Marzluff *et al.* 2012; Cross *et al.* 2013). These techniques allow researchers to repeatedly test the same individuals over time and in response to various stimuli. The results of these studies could provide data about brain activation on a much longer time scale than is currently possible with IEG immunohistochemistry, and could occur in tandem with long-term, repeated behavioural and physiological observations, as these non-invasive techniques do not require euthanasia. Further to this, these techniques could also be employed on wild-caught animals that could then be released into their natural environments and potentially re-captured over time for further testing. As wild-caught animals and ecologically relevant predator stimuli are thought to be the best model for PTSD research (Clinchy *et al.* 2010, 2013; Cohen *et al.* 2012), the use of repeated scans on wild, free-living animals could provide the best possible information to further our understanding of both predator-prey ecology and biomedicine.

In order to fully comprehend the neurobiological impacts of predators on their prey, investigations of behaviour and physiology in tandem with studies of the brain are required. In humans and laboratory mammals, it is understood that the neurobiological impacts of life-threatening fear occur in combination with lasting changes in behaviour and individual physiology (Dielenberg *et al.* 2001; Shiromani *et al.* 2009; Staples *et al.* 2009; Mackenzie *et al.* 2010; Cohen *et al.* 2012). These same investigations should be carried out using wild-caught, non-mammalian individuals. By doing so, researchers can

connect the impacts of fear on the brain to the changes in individual functioning, including physiology, foraging ability, reproductive success, parenting behaviour, and survival overall that we already know to result from predator-induced fear (Lima & Dill 1990; Creel & Christianson 2008; Clinchy *et al.* 2010, 2013; Hawlena & Schmitz 2010; Boonstra 2013). Taken together, the negative direct and indirect effects of predators may explain declines in prey species across the globe. By looking at the indirect effects of predators from an individual to a population level, we will begin to complete the picture of the overarching impacts of predators present in all ecosystems.

4.6 References

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Appendices

Appendix A: Immediate-early gene immunoreactivity in brain regions of interest

Table 1. ZENK immunoreactivity in each treatment in each brain region of interest.

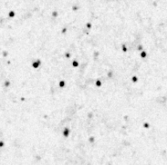
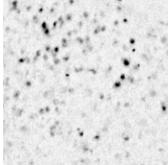
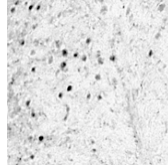
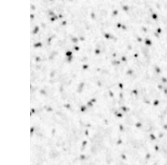
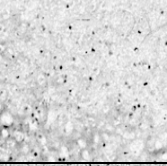
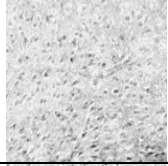
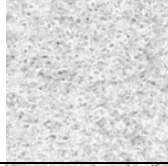
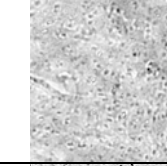
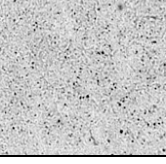
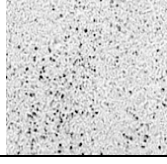
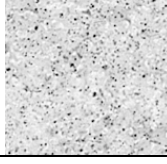
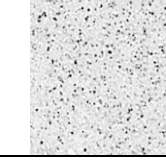
	ZENK			
	control	<i>mobbing call</i>	<i>high zee call</i>	predator
Nucleus taeniae of the amygdala (TnA)				
Hippocampus (Hp)				
Caudal nidopallium (NC)				

Table 2. *c-fos* immunoreactivity in each treatment in each brain region of interest.

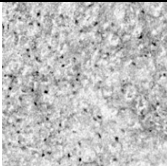
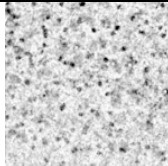
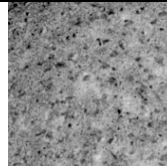
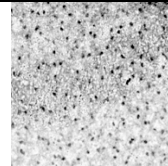
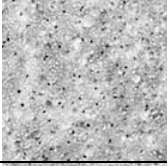
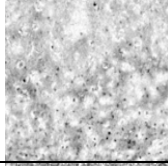
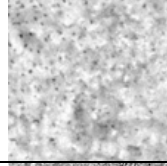
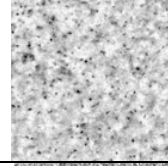




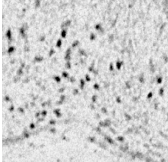
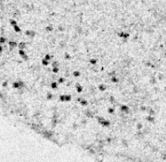
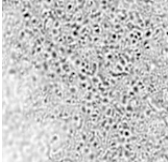
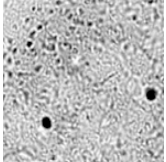
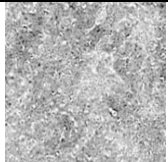
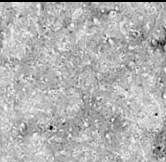
	<i>c-fos</i>			
	control	<i>mobbing call</i>	<i>high zee call</i>	predator
Nucleus taeniae of the amygdala (TnA)				
Hippocampus (Hp)				
Caudal nidopallium (NC)				

Table 3. Δ FosB immunoreactivity in each treatment in each brain region of interest.

	Δ FosB	
	predator	non-predator
Nucleus taeniae of the amygdala (TnA)		
Hippocampus (Hp)		
Caudal nidopallium (NC)		

Appendix B: Playback information

Short-term playbacks

I obtained northern saw-whet owl and red-breasted nuthatch playbacks from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA) and I obtained chickadee *mobbing* and *high zee* alarm calls by exposing wild-caught black-capped chickadees from the University of Western Ontario population to a taxidermic mount of northern saw-whet owls. All sounds were edited in Audacity (Audacity 2.1.0 ®; Mazzoni 2015) and RavenLite (Version 1.0, Cornell University Lab of Ornithology: Bioacoustics Research Program, 2010) to eliminate noise and to shorten calls to the proper length. I organized vocalizations for each treatment into playlists consisting of three calls from three different individuals (i.e. individuals a-b-c; d-e-f etc.) with each call playing for 5 s, followed by 45 s of silence. This 60 s playlist repeated 30 times at 74 dB SPL (following Avey *et al.* 2011). In order to assemble the calls randomly, I assigned a number to each individual sound file and used a random number generator to create lists of three individuals.

Long-term Playbacks

In order to create long-term playbacks to use in my FosB study, I used playlists of multiple predator or non-predator species during daylight for two days. I matched each predator with a non-predator species for maximum amplitude and frequency using RavenLite (Version 1.0, Cornell University Lab of Ornithology: Bioacoustics Research Program, 2010). I obtained all calls from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA) and the Xeno-Canto foundation

(www.xeno-canto.org). I used seven predator species known to prey upon chickadees (Cooper's hawk, *Accipiter cooperii*; American crow, *Corvus brachyrhynchos*; red-tailed hawk, *Buteo jamaicensis*; barred owl, *Strix varia*; sharp-shinned hawk, *Accipiter striatus*; northern saw-whet owl, *Aegolius acadicus*; merlin, *Falco columbarius*), and seven matched non-threatening non-predator species (song sparrow, *Melospiza melodia*; mallard, *Anas platyrhynchos*; blue jay, *Cyanocitta cristata*; northern leopard frog, *Lithobates pipiens*; hairy woodpecker, *Picoides villosus*; wood frog, *Lithobates sylvaticus*; downy woodpecker, *Picoides pubescens*).

I assigned a number to each species within the predator treatment and used a random number generator to determine the order of species for each two-hour playlist. Next, I assigned a number to each individual call, and used a random number generator to randomly select calls from each species in turn to create six unique two-hour playlists for each treatment, which consisted of 110 min of silence with 10 min of calls randomly spaced throughout. I used every species between one and four times (depending on call length and number of calls available for that species) within a treatment in each playlist. I randomized the calls within periods of silence to avoid the possibility of the chickadees habituating to the playback treatments (modified from Zanette *et al.* 2011).

Appendix C: Ethics approval for animal use

AUP Number: 2007-089-08

PI Name: Macdougallshackleton, Scott A

AUP Title: Stress, Development and the Avian Brain

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2007-089-08 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee

Curriculum vitae

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 University of Western Ontario
 London, ON N6A 5B7

Education

M.Sc. (Biology) University of Western Ontario, London, ON	In Progress
H.B.Sc. (Honours Specialization in Biology) University of Western Ontario, London, ON	2013

Research Experience

Graduate Student and Research Assistant University of Western Ontario Department of Biology, Liana Zanette, PhD	2013 – 2015
Fourth Year Honours Research Thesis University of Western Ontario, London, ON	2012 – 2013
Research Volunteer Sinclair Lab, University of Western Ontario, London, ON	2011 – 2012

Teaching and Supervisory Experience

Lab Volunteer Supervisor AFAR animal care and laboratory work	2014 – 2015
Graduate Teaching Assistant Department of Biology, University of Western Ontario, London, ON	
BIOL 1001A/1201A: Introductory Biology	2015
BIOL 3442F: Conservation Biology	2014

Awards and Scholarships

Charlotte Mangum Student Support Program (Society of Integrative and Comparative Biology General Meeting)	2015
Queen Elizabeth II Graduate Scholarship in Science and Technology	2014
University of Western Ontario Biology Graduate Entrance Scholarship	2014
Western Graduate Research Scholarship: Major Scholarship Holder	2014
NSERC Alexander Graham Bell Canada Graduate Scholarship	2013
Ontario Graduate Scholarship	<i>(declined)</i>

University of Western Ontario Biology Graduate Entrance Scholarship	2013
Western Graduate Research Scholarship: Major Scholarship Holder	2013
Edward Barrow and Ida Hodgins Battle Scholarship	2011
University of Western Ontario Continuing Scholarship	2009 - 2013

Conferences and Presentations

May 2015. *Perceived predation risk and the avian brain*. Ontario Ecology, Ethology, and Evolution Colloquium, Toronto, ON.

January 2015. *Perceived predation risk and the avian brain*. Society for Integrative and Comparative Biology Annual Meeting, West Palm Beach, FL, USA.

November 2014. *Quantifying the effects of perceived predation risk on the avian brain*. Friday Philosophicals Graduate Seminar Series (exit seminar), London, ON.

October 2014. *Quantifying the effects of perceived predation risk on the avian brain*. Biology Graduate Research Forum, London, ON.

April 2014. *Quantifying the effects of perceived predation risk on the avian brain*. Friday Philosophicals Graduate Seminar Series (entrance seminar), London, ON.

April 2013. *Fear on the brain: using immediate-early gene expression to identify the avian fear centre*. Western Biology Day 2013, London, ON.

March 2013. *Fear on the brain: using immediate-early gene expression to identify the avian fear centre*. Ontario Biology Day 2013, Hamilton, ON.

Current Certifications

National Lifeguard	2015
Worker Health and Safety Awareness	2014
Whitewater Rescue Technician III	2014
General Laboratory Safety and Hazardous Waste Management	2013
Wilderness First Responder	2012
Comprehensive Workplace Hazardous Materials Information Systems	2011

Professional Memberships

Society for Integrative and Comparative Biology