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The Enduring Effects of Fear on the Brain and Behaviour in Wildlife

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Abstract

Predators affect prey both through direct consumption and through non-consumptive fear effects. Predator induced fear has been shown to alter prey behaviour, where increased vigilance and reduced foraging can scale up to population and community level consequences. In this thesis, I used laboratory model studies for post-traumatic stress disorder (PTSD) as a template to study the neural mechanisms underlying the enduring behavioural response to fear in wildlife. I used audio playbacks in three manipulations in increasingly natural conditions. In all manipulations, playbacks simulated either the presence of predators or non-predator controls. Playbacks were then silenced for one week at which time I tested for enduring effects of predator induced fear in three key brain regions associated with fear processing. In Chapter 2, I quantified the enduring effects of fear on neurogenesis in laboratory housed black-capped chickadees (*Poecile atricapillus*). I found sex-dependent effects on hippocampal cell proliferation and suppression of immature neurons in the medial ventral arcopallium (avian homologue to the amygdala) for both sexes, consistent with an enduring memory of fear. In Chapter 3, I manipulated fear again in black-capped chickadees but this time they were living outside in semi-natural conditions. I quantified the effects of fear on foraging behaviour, activation, neurogenesis, and neuron structure. Only neuron structure demonstrated enduring effects of fear which included longer dendrites in both the hippocampus and caudolateral nidopallium (avian analogue to the prefrontal cortex) and increased dendritic branching in the caudolateral nidopallium. In Chapter 4, I manipulated fear in free-living meadow voles (*Microtus pennsylvanicus*). I report enduring, sex dependent, effects of fear on neural activation, neurogenesis, and apoptosis, and enduring effects of fear on neuron structure in both sexes. Enduring effects on neurobiology were accompanied by a behavioural shift in nocturnal activity whereby voles who heard predators one week earlier were caught later in the night. Overall, my thesis demonstrates that wildlife show enduring, PTSD-like effects on neurobiology in response to predator-induced fear and that these effects are often sex dependent. Demonstrating PTSD-like effects on the brains of wild animals suggests that predator-induced fear may have adaptive value for surviving future predator encounters.

Keywords

predator-prey interaction, fear, post-traumatic stress disorder, fear memory, neurogenesis, Δ FosB, apoptosis, dendritic morphology

Summary for Lay Audience

Predators affect prey by eating them, but the fear of becoming dinner can also have lasting effects. While critical for wildlife survival, fear can be costly as they may have to miss a meal to avoid becoming a meal. I used research on post-traumatic stress disorder (PTSD) as a template to measure the long-lasting effects of fear on the brain and behaviour in wildlife, as PTSD can last a lifetime after even a single life-threatening event. In this thesis, I used sounds of local predator and non-predator species to measure the long-lasting effects of fear in brain regions known to respond to fear. My first chapter measured the effects of fear on newly formed brain cells in black-capped chickadees living in the lab. Males had fewer newborn brain cells, while both sexes had fewer young neurons, when scared. My second chapter looked at how black-capped chickadees respond to fear when living in large outdoor aviaries. Here I measured the long-lasting effects on newly formed brain cells, brain activation, and brain cell structure, and found that one week later the tree-like branches of brain cells (dendrites) got longer and grew more branches when birds were scared. Finally, I measured the effects of fear in a wild rodent species (meadow voles) in their natural habitat. I showed that fear led to changes in newly born brain cells, brain activation, and brain cell death, with males and females showing opposite effects in the brain. I also found that brain cells got longer and had more branches (dendrites) in both male and female voles. Overall, I show how fear can induce long lasting changes in the brain that differ between males and females, suggesting fear memories may be a sign of a healthy mind and have advantages for survival.

Co-Authorship Statement

This dissertation contains three data chapters that are currently in preparation for publication, of which I will be the lead author. All data chapters were completed under the supervision of Dr. Liana Zanette, with support from Dr. Michael Clinchy, Dr. Scott MacDougall-Shackleton, and Dr. Craig Bailey.

A version of Chapter 2 has been submitted for publication to *Integrative Organismal Biology* (IOB-2025-030.R1) and is currently awaiting a final editorial decision pending minor revisions. Sarah Davidge, Emma C. Hobbs, Scott A. MacDougall-Shackleton, Michael Clinchy, and Liana Y. Zanette will be co-authors. All authors conceived the study, E.C.H. conducted the field work, L.E.W. and S.A.M.-S. developed and performed the immunohistochemistry assays, L.E.W. and S.D. performed the microscopy and image analysis, L.E.W, S.D., and L.Y.Z analyzed the data, and L.E.W and L.Y.Z. drafted the manuscript with editorial contributions from all other authors. Funding for this chapter was provided by L.Y.Z. and S.A.M.-S..

Chapter 3 is in preparation for peer review and publication, with Brielle Comartin, Scott A. MacDougall-Shackleton, Craig D. C. Bailey, Michael Clinchy, and Liana Y. Zanette as co-authors. All authors conceived the study, L.E.W. conducted the field work, L.E.W., B.C., and S.A.M.-S. developed and performed the immunohistochemistry assays, L.E.W. and C.D.C.B developed and performed the Golgi-Cox protocols, L.E.W., and B.C. performed the microscopy and image analysis, L.E.W., B.C., and L.Y.Z analyzed the data, and L.E.W and L.Y.Z. drafted the manuscript with editorial contributions from all other authors. Funding for this chapter was provided by L.Y.Z., S.A.M.-S., and C.D.C.B..

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List of Abbreviations and Symbols

AFAR	Advanced Facility for Avian Research
Amg	Amygdala
AMV	Medial ventral arcopallium
ANOVA	Analysis of variance
BA	Basal amygdala
BLA	Basolateral amygdala
BrdU	5-bromo-2'- deoxyuridine
CA1	CA1 layer of the cornu ammonis
CA3	CA3 layer of the cornu ammonis
CeA	Central Amygdala
CG1	Anterior cingulate cortex
DCX	Doublecortin
DG	Dentate gyrus
ESW	Environmental Sciences Western
Hp	Hippocampus
IL	Infralimbic area
LA	Lateral amygdala
LSD	Fisher's least significant difference
NCL	Caudolateral nidopallium
PCNA	Proliferating cell nuclear antigen
PFC	Prefrontal Cortex
PrL	Prelimbic area
PTSD	Post traumatic stress disorder

Chapter 1

1 General Introduction

Predator-prey dynamics have traditionally focused on direct killing alone, as predation represents the ultimate, negative effect on individual fitness since dead prey do not reproduce (Kavaliers and Choleris 2001, Boonstra 2013). However, limited capture success leads predators to scare more prey than they kill (Vermeij 1982). To survive, animals must be able to perceive predation risk and respond accordingly. Many aquatic species develop inducible morphological defenses that are costly, long term and/or permanent, including defensive structures such as helmets in *Daphnia* (Dodson 1989), and changes in body morphology such as tadpoles (*Rana pirica*) exhibiting the bulgy morph (Kishida and Nishimura 2004). Predation risk has also been shown to induce changes in physiology and behaviour in many taxa, such as increased glucocorticoid hormones in many species, including brown lemmings (*Lemmus trimucronatus*) and collared lemmings (*Dicrostonyx groenlandicus*; Fauteux et al. 2018) and snowshoe hares (*Lepus americanus*; Sheriff et al. 2009) and increased body and fat mass in brown-headed cowbirds (*Molothrus ater*), with a corresponding change in escape behaviour (Walters et al. 2017). When behavioural changes result in reduced parental provisioning, anti-predator behaviour can also scale up to population level impacts due to reduced offspring survival (Zanette et al. 2011, Dudeck et al. 2018, Allen et al. 2022). In this chapter I will review the literature on the enduring effects of predator-induced fear on the brain in wild animals, the use of predator-induced fear in animal models for post-traumatic stress disorder, the brain regions involved in the fear processing pathway, and neural markers used to quantify the enduring effects of predator-induced fear on the brain.

1.1 Predator-prey interactions and the ecology of fear

The *ecology of fear* describes the population, community, and ecosystem level impacts arising from individual behavioural responses when faced with the threat of predation (Brown et al. 1999, Brown 2019, Zanette and Clinchy 2020). At the community level, the fear of predators has been demonstrated to cause trophic cascades,

with reduced mesocarnivore feeding leading to increases in the density of their prey and decreases in the density of their prey's prey (Suraci et al. 2016). Changes to individual anti-predator behaviour can also be seen across the *landscape of fear*, where prey alter their movement across the landscape to avoid predator encounters (Laundré et al. 2001, 2010, Gaynor et al. 2019). For animals with advanced cognition, the landscape of fear is thought to exist as a mental map used to proactively avoid predators by altering habitat use (Creel 2018). The behavioural changes in wildlife described by the ecology of fear and across the landscape of fear imply that prey retain an enduring memory of previous predator encounters to reduce the likelihood of future predator encounters (Zanette and Clinchy 2017).

While there is abundant evidence that predators can alter the behaviour and physiology of wild prey, very little is known about the enduring effects of predators on the brain in wild animals. The existing literature was compiled using Web of Science, with the search cues “predat*”, “fear”, “brain”, and “wild” in various combinations to find all relevant papers that measured the effects of predator-induced fear on the brain in either wild caught animals or the laboratory reared offspring of wild caught animals. This search led to 12 papers that looked at the effects of predators or their cues on either wild caught animals or the laboratory reared offspring of wild animals. These are outlined for the effects of predators or their cues on the brain in wild caught animals in Table 1.1, while the effects on the laboratory reared offspring of wild animals are outlined in Table 1.2. Observational studies have demonstrated that guppies (*Poecilia reticulata*) living in environments with naturally high predator pressure have larger brains, altered brain anatomy, and increased vasotocin gene expression (Kotrschal et al. 2017, Reddon et al. 2018, 2022, Reyes et al. 2022). Electric fish (*Brachyhypopomus occidentalis*) and Trinidadian killifish (*Rivulus hartii*) living in naturally high predator environments have also been demonstrated to have reduced brain cell proliferation compared to those living in rivers with low predation pressure (Dunlap et al. 2016, 2019). Wild caught animals have also been bred in laboratory conditions, with their offspring used in experimental manipulations of predator induced fear. This has been demonstrated to increase Δ FosB expression in Brandt's voles (*Lasiopodomys brandtii*) (Hegab et al. 2014), reduce dendritic length and branching in gerbilline species (Cohen et al. 2023), reduce cell

proliferation in electric fish (Dunlap et al. 2017), and increase serotonin and 5-hydroxyindoleacetic acid in sea trout (*Salmo trutta*; Lepage et al. 2000). To date, the only experimental manipulations quantifying the enduring effects of fear on the brain of wild caught animals have been conducted in birds, with black-capped chickadees (*Poecile atricapillus*) showing increased Δ FosB activation and brown-headed cowbirds (*Molothrus ater*) showing suppressed neurogenesis and alterations to dendritic morphology one week after hearing predator calls (Witterick 2017, Zanette et al. 2019). Of the papers looking at the effects of predators or their cues on the brain in wild animals, all of the observational studies on wild caught animals were done in fish while the only two manipulations were done with birds. For the laboratory reared offspring of wild caught animals, the literature is split between experiments on fish and mammals. While there are still many unanswered questions about the enduring effects of fear on the brain in wild animals, there is a large body of literature examining fear effects in lab rodents where live predators or their cues (such as calls, mounts, or odours) are used to study post-traumatic stress disorder (PTSD; Deslauriers et al. 2018).

1.2 Post-traumatic stress disorder

Predator stress, using either live predators or their cues, is a commonly used tool for inducing PTSD-like symptoms in laboratory rodent models, providing a valuable tool to understand the neural mechanisms underlying the enduring and intrusive memories experienced after a life threatening experience (Deslauriers et al. 2018). Using predator stress as the threatening stimulus adds etiological validity to PTSD animal models, while producing robust behavioural phenotypes evident after a single stressful experience (Deslauriers et al. 2018). In humans, symptoms are required to persist for at least 30 days to meet the criteria for PTSD (American Psychiatric Association 2013). To qualify as an animal model for PTSD effects must endure for at least 7 days in a small bodied animal, accounting for the animal's shorter lifespan (Deslauriers et al. 2018). In a small bodied animal, enduring effects of fear lasting 7 days could have lasting demographic consequences if this leads to altered parental behaviour during key phases of offspring development. For example, black-capped chickadees begin to open their eyes by day 7 post hatching, and fledge from the nest by day 16 (Smith 1991). In meadow voles

(*Microtus pennsylvanicus*), weaning occurs at 14 days after birth (Kurta 2017). This can lead to lasting demographic consequences if parents alter behaviour for 7 days due to predation risk and reduce provisioning to their offspring (Zanette et al. 2011, Dudeck et al. 2018).

When using laboratory animal models to understand neural mechanisms with the goal of extrapolating to other taxa, either for wildlife or human applications, experimental subjects should be representative of the population and include both sexes. Laboratory animal models for PTSD have traditionally used only male experimental subjects, raising questions about the enduring effects of fear on the female brain, while the limited data available suggest that females may actually be more susceptible to the predator stress paradigm than males (Deslauriers et al. 2018). In humans, it is also women that are more likely to develop PTSD, with a lifetime prevalence that is more than twice that of men (Duncan et al. 2018). Moreover, understanding how both sexes respond to predator-induced fear is also ecologically important as it is the survival and fecundity of females that affect reproductive rates and therefore population growth. This suggests that including both male and female subjects in future experimental manipulations is critical to understanding the enduring, PTSD-like effects of predator-induced fear on the brain.

While laboratory animal models for PTSD offer many benefits to increasing our understanding of the etiology of PTSD due to the ability to control the experimental environment and manipulate the exposure to life-threatening stimuli, they must also account for the possibility that captivity may be playing a role in the neural response to fear. Given that immobilization and restraint stress are also commonly used to induce PTSD-like symptoms (Deslauriers et al. 2018), it is possible that captivity may be interacting with the fear of predators to synergistically amplify the enduring effects of fear seen under laboratory conditions. Captivity may amplify the effects of fear due to a lack of available refugia in which to hide or due to an inability to escape from the cage to an area that is perceived to be lower risk, as an animal may do in the wild. In order to understand the role that predator-induced fear plays in the observed PTSD-like effects on the brain and behaviour in animal models, it is necessary to conduct experimental

manipulations under increasingly natural conditions to minimize any potential confounding influence of captivity.

While PTSD is generally treated as a maladaptive disorder in humans, it has been suggested that there may be evolutionarily adaptive value to retaining these enduring memories if it promotes survival in future life-threatening encounters (Cantor 2009, Clinchy et al. 2011, Diamond and Zoladz 2016, Cohen et al. 2023). Finding PTSD-like enduring effects of predator induced fear in wildlife exposed to cues of their natural predators would provide support that PTSD is a natural phenomenon that may be evolutionarily adaptive, which may help to reduce the stigma for humans suffering from PTSD. Animal models for PTSD provide us with neural pathways involved in fear processing and neural markers that have shown enduring effects on the brain, necessary tools for new research directions to address concerns over whether the observed enduring effects on neurobiology demonstrated in mostly male laboratory rodents can be generalized to both sexes and in their wild counterparts (Clinchy et al. 2011, Cohen et al. 2023).

1.3 Brain Regions in the Fear Processing Pathway

Animal models studying PTSD and other aspects of the fear response provide an extensive literature on the neural circuits involved in the fear processing pathway in mammals. Three key brain regions pertinent to the mammalian fear processing pathway are the amygdala, hippocampus, and prefrontal cortex (Figure 1.1; Gross and Canteras 2012, McEwen et al. 2016, Izquierdo et al. 2016, Lambert and McLaughlin 2019). While the neural circuitry of the fear processing pathway extends beyond these three regions, they are most implicated for their roles in the enduring effects of fear associated with PTSD.

The mammalian amygdala is thought to act as a switchboard, conveying information about predator stress to other brain regions (Gross and Canteras 2012). The amygdala, particularly the basolateral amygdala and central amygdala, is the terminus for sensory inputs and is essential for the acquisition and expression of conditioned fear, particularly auditory fear conditioning (Tovote et al. 2015, Silva et al. 2016). A

hyperfunctional amygdala is suggested to play a key role in the hypervigilance demonstrated in people suffering from PTSD (Diamond and Zoladz 2016). The medial ventral arcopallium is proposed to be the avian homologue to the mammalian amygdala, and has been demonstrated to show enduring, PTSD like effects on Δ FosB activation and neurogenesis in songbirds exposed to predator calls (Yamamoto et al. 2005, Witterick 2017, Mello et al. 2019, Zanette et al. 2019).

The mammalian hippocampus is involved in the encoding and retrieval of fear memory and spatial memory, with direct projections to the amygdala (Gross and Canteras 2012, Izquierdo et al. 2016). The hippocampus also plays an important role in incorporating contextual cues from the environment with threatening or painful stimuli (Gross and Canteras 2012, Tovote et al. 2015). Adult humans with PTSD commonly show reduced hippocampal volume when compared to those without PTSD or trauma exposure (Lambert and McLaughlin 2019). The avian hippocampus is proposed to be a functional homologue of the mammalian hippocampus, with previous work demonstrating enduring effects of fear including increased Δ FosB activation, suppressed neurogenesis, and sex dependent alterations to dendritic morphology in songbirds exposed to predator calls (Colombo and Broadbent 2000, Witterick 2017, Zanette et al. 2019).

The mammalian prefrontal cortex plays an important role in working memory, executive functions, and decision making (Arnsten 2009, Dixsaut and Gräff 2021). Acute, uncontrollable stress has been shown to impair cognitive functions mediated by the prefrontal cortex in both humans and animals (Arnsten 2009). Reciprocal interactions between the amygdala and prefrontal cortex have also been suggested to be associated with successful stimulus discrimination after fear conditioning (Tovote et al. 2015). The caudolateral nidopallium is proposed to be the avian homologue to the prefrontal cortex, and has been demonstrated to play an important role in learning-induced neuronal plasticity, along with working memory and decision making (Güntürkün 2005, Herold et al. 2011, Li et al. 2020, Parishar et al. 2024).

1.4 Neural Markers

To quantify the enduring effects of fear on the brain, it is necessary to label markers that would be subject to stress induced plasticity in response to predator-induced fear. For this thesis, I have focused on markers that have previously been demonstrated to label enduring effects of fear on neuronal activation, neurogenesis, apoptosis, and neuron structure in laboratory models.

Neuronal activation can be measured using immediate early gene (IEG) expression. IEG expression is typically quick and transient, with unstable IEG protein products. FOS and JUN are two widely used IEGs that are rapidly and transiently induced by a variety of stimuli such as stress, labelling neurons that were recently depolarized, and play a key role in cellular events including proliferation, differentiation, and survival (Nestler 2015, Bahrami and Drabløs 2016). FOS proteins heterodimerize with JUN proteins to form active transcription factor activator proteins that are rapidly induced in neurons that were recently depolarized and have a half-life in mammals ranging from minutes to a couple hours (Nestler 2015, Bahrami and Drabløs 2016, Robison and Nestler 2022). While most IEGs are transient, an enduring marker of earlier activation can be measured using Δ FosB, an immediate early gene transcription factor from the FOS family that is highly stable and persists in the brain due to a half-life of up to 8 days (Robison and Nestler 2022). While Δ FosB is widely studied for its role in the reward circuitry of the brain and the associated implications for addiction, induction of hippocampal Δ FosB has also been linked to stress resilience, learning, and memory (Robison and Nestler 2022). Enduring increases in Δ FosB in response to predator induced fear have been reported in both rodents and songbirds in the laboratory (Staples et al. 2009, Mackenzie et al. 2010, Zanette et al. 2019). Throughout this thesis, all results for activation will be referring to the enduring marker of earlier activation as measured through Δ FosB immunoreactivity.

Neurogenesis is a process consisting of both cell proliferation, or the birth of new cells, and survival of these new cells to maturity (Galea et al. 2006). Adult neurogenesis is widely studied in both rodents and birds, particularly for the role of neurogenesis in memory formation and forgetting (Barnea and Pravosudov 2011, Frankland et al. 2013).

Neurogenesis can be assessed at different stages of the cell cycle, including proliferation, differentiation, immature cells, and mature neurons. Commonly used markers include 5-bromo-2'-deoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA), and Ki-67 as markers of cell proliferation, doublecortin (DCX) as a marker of immature neurons, and neuron-specific nuclear protein (NeuN) as a marker of mature, postmitotic cells (von Bohlen und Halbach 2011). The two markers of interest for this thesis are proliferating cell nuclear antigen (PCNA) and immature neurons with doublecortin (DCX). PCNA is a subunit of DNA polymerase-delta that is present during all phases of the cell cycle with the highest expression during the G₁ and S-phases (von Bohlen und Halbach 2011). As an essential component of DNA replication and the repair of DNA errors, PCNA can be used as a marker for adult neurogenesis, with a half-life of 20 h in rodents and 8-31 h in human cells (Bravo and Macdonald-Bravo 1987, Stewart and Dell'orco 1992, Sasaki et al. 2009). PCNA has also been used to label brain cell proliferation in birds and in fish (Dunlap et al. 2016, 2017, 2019, Mazengenya et al. 2017, 2018, 2020, Diez et al. 2021). DCX is a microtubule associated protein expressed in migrating neuronal precursors, with expression starting from the development of neuroblasts and downregulated as the mature neuronal marker NeuN appears (Couillard-Despres et al. 2005). DCX is a widely used marker of neurogenesis, verified in both songbirds (Balthazart and Ball 2014) and rodents (Couillard-Despres et al. 2005)

The suppression of neurogenesis has been demonstrated to promote memory persistence by preventing the replacement of existing memories (Frankland et al. 2013, Akers et al. 2014). After experiencing chronic stress, experimentally elevated neurogenesis was demonstrated to attenuate anxiety-like behaviour, suggesting that suppressed neurogenesis has an enduring, PTSD-like effect on memory (Fujikawa et al. 2024). Drug induced suppression of neurogenesis has also been shown to prolong the anxiety-like behaviours in rats following a PTSD-like stressor (Schoenfeld et al. 2019). Suppressed neurogenesis has been shown in response to predator cues both in lab rodents (Tanapat et al. 2001, Vignisse et al. 2017, Wu et al. 2019) and in captive brown-headed cowbirds under semi-natural conditions (Witterick 2017). Additionally, fish living in naturally low predator environments showed greater levels of cell proliferation than

conspecifics living in habitats with naturally high predator populations (Dunlap et al. 2016).

Apoptosis has been quantified in mammals using activated caspase-3, an executioner caspase that is activated in apoptotic cells. It is a critical mediator of both the intrinsic and extrinsic pathways to apoptosis and essential for neuronal cell death (McIlwain et al. 2015, Asadi et al. 2022). Activated caspase-3 is also a critical mediator of synaptic plasticity and neurodevelopment, as synaptic connections are altered and unnecessary neurons are eliminated (Asadi et al. 2022). Elevated apoptosis in the hippocampus, amygdala, and prefrontal cortex has been reported in response to stress in rodent models for PTSD (Ding et al. 2010, Li et al. 2010, Xiao et al. 2011, Yu et al. 2014, Jia et al. 2018, Han et al. 2021). While apoptosis has been labelled extensively in rodent models, there is currently no marker that reliably labels activated caspase-3 in the avian brain (MacDougall-Shackleton et al, unpublished data).

The enduring effects of predator-induced fear on neuron structure can be measured by alterations to dendritic morphology. Dendrites are functionally specialized to receive signals and are highly plastic, altering their length and branching to modify synaptic connectivity (Kulkarni and Firestein 2012). Structural changes in the adult brain, such as through dendritic remodeling, are often implicated in response to stress (reviewed in McEwen et al. 2012, 2016, Chattarji et al. 2015, Conrad et al. 2017). Alterations to dendritic morphology in response to predators or their cues have been demonstrated in mammals in the hippocampus (Hoffman et al. 2016, Cohen et al. 2020, 2023) and basolateral amygdala (Mitra et al. 2009, Adamec et al. 2012), while comparable effects have been demonstrated in the prefrontal cortex in response to restraint stress (Liston et al. 2006, Radley et al. 2006, Garrett and Wellman 2009) in laboratory rodents. Alterations to neuron structure in response to chronic mild stress have also been demonstrated in the avian hippocampus (Arya et al. 2024), suggesting that these effects may be conserved across taxa.

In this thesis, I focus on the enduring effects of fear in the amygdala, hippocampus, and prefrontal cortex, along with their avian counterparts the hippocampus,

medial ventral arcopallium, and caudolateral nidopallium. In the avian brain, I quantified the enduring effects of fear on PCNA and DCX in Chapters 2 and 3, with Δ FosB and dendritic morphology also measured in Chapter 3. In the mammalian brain in Chapter 4, I quantified the enduring effects of fear on Δ FosB, DCX, activated caspase-3, and dendritic morphology.

1.5 Study Species

1.5.1 Black-capped Chickadee

The study species used for Chapters 2 and 3 is the black-capped chickadee (*Poecile atricapillus*; hereafter chickadees), a small bodied, nonmigratory songbird that is a year-round resident throughout Canada and the Northern United States. Chickadees were an ideal study species for these chapters as they are locally abundant and adapt easily to group housing in captivity due to the formation of social flocks with highly stable dominance hierarchies in the fall and winter (Smith 1991). Chickadees are widely used as an avian model species to study memory and the avian hippocampus, particularly in relation to seasonal changes in food storing behaviour (Sherry and Hoshooley 2010, Sherry and MacDougall-Shackleton 2015). Chickadees were selected for these chapters to expand on a previous experiment demonstrating enduring effects of fear on activation and hypervigilance behaviour in a laboratory setting. One week after hearing predator calls in the lab, chickadees spent more time immobile immediately after hearing a conspecific alarm call, the high zee, and showed increased Δ FosB in both the Hp and AMV (Zanette et al. 2019). Using chickadees for both Chapters 2 and 3, along with the results from the previous laboratory experiment, allowed for comparisons between the fear response in increasingly natural conditions from the laboratory to a semi-natural environment through the use of the same neural markers within the same study species.

1.5.2 Meadow Vole

For Chapter 4, I worked on completely free-living meadow voles (*Microtus pennsylvanicus*), a small bodied rodent present throughout Canada and across the North and Eastern areas of the United States (Kurta 2017). Meadow voles prefer moist grassy fields, and are primarily crepuscular, although they may be active at any time of day or

night (Kurta 2017). Meadow voles were an ideal species for this chapter due to their population density (100-200 per hectare; Saunders 1988) and relatively small home range (less than 0.3 ha) (Kurta 2017). The short gestation period (21 days), large litters, and rapid growth to sexual maturity (4-5 weeks) maintains meadow vole populations despite high rates of natural predation (Kurta 2017), allowing for minimal experimental impact on the wild population numbers. Meadow voles are also a widely used model species in neurobiology, due to their highly conserved neuroanatomy, increased genetic and behavioural variation when compared to laboratory bred rats and mice, and unique social structures (Kenkel et al. 2021).

1.6 Thesis Overview

Overall, this thesis will address the enduring effects of fear on the brain and behaviour in wild animals at varying levels of captivity. In Chapter 2, I quantify the enduring effects of fear on neurogenesis in wild caught chickadees under laboratory conditions, building on previously reported increased activation and hypervigilance behaviour under the same experimental conditions (Zanette et al. 2019). In Chapter 3, I replicated a protocol previously used to examine predation risk in cowbirds (Walters et al. 2017, Witterick 2017) and experimentally manipulated predation risk with chickadees living under semi-natural conditions in large outdoor aviaries and measured the effects on foraging behaviour, neuron structure, neurogenesis, and activation. The semi-natural conditions exposed chickadees to natural variation in fall environmental conditions while protecting them from direct predation and allowing natural refuge-seeking behaviours. In my MSc I used the same protocol to scare brown-headed cowbirds and found suppressed neurogenesis in the avian homologues to the hippocampus and amygdala, along with increased dendritic length and branching in the hippocampus of female birds (Witterick 2017). This chapter expands on both Chapter 2 and previous work from my MSc by measuring the enduring effects of fear on the brain in chickadees under increasingly natural conditions. In Chapter 4, I expanded upon my earlier work in the laboratory and semi-natural conditions and conducted a predation risk manipulation with free-living voles, quantifying the enduring effects of fear on activation, neurogenesis, apoptosis, and neuron structure. Working with wild voles in their natural habitat allowed me to address

the enduring effects of fear while removing any potential influence of captivity. Finally, Chapter 5 will address my results in the general discussion, how the different markers measured compare across increasingly natural conditions, and potential future research directions for the enduring effects of fear on the brain and behaviour in wildlife.

Table 1.1: Literature on the effects of predators on the brain in wild animals

Taxa	Reference	Method	Observation or manipulation?	Lab or Field?	Measure	Result from high predation treatment?
Fish	Dunlap et al. 2016	Natural variation in predator density	Observation	Field	Forebrain cell proliferation	Decreased density of PCNA+ cells in high predation environment
Fish	Kotrschal et al. 2017	Predation pressure estimated from mean predator biomass	Observation	Field	Whole brain size, brain region size	Increased predator biomass led to larger brain, telencephalon, and optic tectum, smaller olfactory bulbs and hypothalamus
Fish	Reddon et al. 2018	Natural presence or absence of predators	Observation	Field	Brain mass	Males living in environment with predators had heavier brains; no difference in females
Fish	Dunlap et al. 2019	Natural presence or absence of predators	Observation	Field	Cell proliferation in midbrain and forebrain	Increased cell proliferation in midbrain and forebrain
Fish	Reddon et al. 2022	Natural presence or absence of predators	Observation	Field	Whole brain vasotocin and isotocin expression	Increased vasotocin expression, no difference in isotocin expression
Fish	Reyes et al. 2022	Natural presence or absence of predators	Observation	Field	Brain weight and volume	Brain weight increases with predation level, females brains heavier than males
Bird	Witterick 2017	Predator playbacks	Manipulation	Field	Immature neurons (doublecortin), dendritic morphology in hippocampus (Hp) and medial ventral arcopallium (AMV)	Suppressed immature neurons, longer in both Hp and AMV, more branched dendrites in Hp of female birds
Bird	Zanette et al. 2019	Predator playbacks	Manipulation	Lab	Δ FosB in Hp and AMV	Increased Δ FosB in both Hp and AMV

Table 1.2: Literature on the effects of predators on the brain in laboratory reared offspring of wild animals

Taxa	Reference	Method	Observation or manipulation?	Measure	Result from high predation treatment?
Fish	Lepage et al. 2000	Predator presence	Manipulation	Serotonin and 5-hydroxyindoleacetic acid (5-HIAA)	Reduced serotonin in telencephalon, increased 5-HIAA in telencephalon, midbrain, and brain stem
Fish	Dunlap et al. 2017	Simulated predator exposure (light taps on tail), tail amputation	Manipulation	Cell proliferation (PCNA) in telencephalon	Reduced PCNA+ cells after both simulated predator exposure and tail amputation
Mammal	Hegab et al. 2014	Predator odour	Manipulation	Δ FosB in hypothalamus	Increased Δ FosB mRNA expression
Mammal	Cohen et al. 2023	Predator playbacks	Manipulation	Dendritic morphology in the dentate gyrus	Reduced dendritic length and number in animals who showed greatest behavioural response to predator calls

1.7 References

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Chapter 2

2 Fear of predators suppresses neurogenesis in the brains of wild songbirds

2.1 Introduction

Fear of predation is a ubiquitous experience of wildlife, and can cause enduring effects on prey long after the immediate threat has passed, wherein memories of the life-threatening, traumatic experience of surviving a predator encounter persist (Zanette and Clinchy 2020). An enduring memory of past predator encounters fundamentally underlies the *ecology of fear*. Here, ecologists look beyond the immediate and transient stress response and focus on understanding how increased vigilance and reduced foraging of frightened animals can lead to lasting behavioural changes with population and community level consequences (Brown et al. 1999, Zanette and Clinchy 2019, 2020, Brown 2019, Gaynor et al. 2021). While it is implied that enduring effects in terrestrial wildlife are due to prey retaining a memory of predator-induced fear, the effect on the brain has never been explicitly tested (Zanette and Clinchy 2017). While little is known about the enduring effects of predator-induced fear on the brains of wildlife, there is a large body of literature examining fear effects in lab rodents where live predators or their cues (such as calls, mounts, or odours) are used to study post-traumatic stress disorder (PTSD) (Deslauriers et al. 2018).

Predator stress, through either predator animals or their cues, is a commonly used technique for inducing PTSD-like symptoms in animal models due partly to the robust behavioural phenotypes produced and partly because such phenotypes can be produced after a single stressful experience, providing a valuable tool to understand the neural mechanisms behind the persistent and intrusive memories remaining after a life threatening event (Deslauriers et al. 2018). To distinguish PTSD-like effects from an immediate, transient anti-predator response, PTSD animal models also indicate that effects must endure for at least 7 days in a small bodied animal, such as rodents or songbirds (Deslauriers et al. 2018). The existing PTSD animal model literature focuses heavily on male animals, raising many questions about the enduring effects of predator-

induced fear on female brains (Deslauriers et al. 2018). Limited data are available on female animals but females may be more susceptible to predator-induced fear than males, at least in rodents (Deslauriers et al. 2018). Sex differences in selection pressures may incur additional fitness costs (Connallon et al. 2010). Moreover, understanding how both sexes respond to predator-induced fear is also ecologically important as it is the survival and fecundity of females that affects reproductive rates and therefore population growth. Assessing neural PTSD-like effects in both sexes and in a wild animal are necessary next steps in addressing concerns over whether the observed effects in the neurobiology of lab animal models can be generalized to their wild counterparts (Clinchy et al. 2011, Cohen et al. 2023).

While an enduring and disruptive memory of a traumatic event is a known symptom of PTSD, an enduring memory of how to anticipate and survive life threatening situations in a dynamic and changing environment would provide a fitness advantage for wildlife (Cohen et al. 2023). Behavioural changes in response to predators imply that prey animals are able to retain a memory of previous predator encounters as they alter their behaviour to avoid future predator encounters (Brown et al. 1999, Zanette and Clinchy 2020). Experimental evidence in the laboratory indicates that the ability to retain fear memories alters anti-predator behavioural responses, which increase the likelihood of surviving future predator encounters (Crook et al. 2014, Zhong et al. 2023). This enduring effect of predator-induced fear may be most clearly seen in the concept of the *landscape of fear*, wherein the spatial variation in predation risk leads to alterations in prey movement across a landscape (Laundré et al. 2001, 2010, Gaynor et al. 2019). These proactive (Creel 2018) responses imply that animals are able to retain a memory of where they previously encountered predators in addition to the types of habitat where they might expect to encounter predators again (Suraci et al. 2019, Epperly et al. 2021, Clermont et al. 2021). Similarly, wildlife are well known to mount reactive anti-predator behavioural responses once an encounter with a predator has begun (Creel 2018), implying that prey retain a memory of their predators. If wild animals are forming enduring memories of predator experiences, I would expect to see an enduring signature in the brain indicative of memory retention.

If animals retain an enduring memory of fear, I might expect to see alterations to adult neurogenesis. The suppression of neurogenesis in the hippocampus promotes memory persistence, likely by preventing the replacement of existing memories (Frankland et al. 2013, Akers et al. 2014). For example, increasing neurogenesis through exercise has been shown to weaken the memory of a trauma and can reduce PTSD-like behaviours in lab mice (Fujikawa et al. 2024). Further, in mice displaying elevated anxiety-like behaviours in response to chronic stress, suppressed neurogenesis impaired recovery (Schoenfeld et al. 2019), implying that suppressed neurogenesis would indicate an enduring, PTSD-like memory of fear. Similarly, increased neurogenesis has been shown to weaken existing spatial memories (Epp et al. 2016), suggesting that suppressed neurogenesis may play an important role in maintaining the mental map that wildlife need to proactively (*in sensu* Creel 2018) respond to variations across the landscape of fear. I have previously demonstrated that fear of predators induced enduring effects on activation and hypervigilance behaviour in wild-caught birds lasting at least one week after hearing predator vocalizations, which is consistent with an enduring memory of predator fear (Zanette et al. 2019). Therefore, I would expect to see a corresponding suppression in neurogenesis in birds under the same experimental conditions.

To experimentally test if predator-induced fear causes enduring changes in neurogenesis in wild animals, I followed methodology from both animal models for PTSD and the ecology of fear. I measured two stages of neurogenesis, quantifying both cell proliferation using proliferating cell nuclear antigen (PCNA), and immature neurons using doublecortin (DCX; von Bohlen und Halbach 2011). Following research using animal models for PTSD, I looked for effects enduring at least 7 days, which is considered an enduring effect in a small bodied animal (Deslauriers et al. 2018), and in three brain regions (the avian counterparts to the mammalian hippocampus, amygdala, and prefrontal cortex) most implicated in fear processing and PTSD (Diamond and Zoladz 2016, Akiki et al. 2017, Deslauriers et al. 2018, Lambert and McLaughlin 2019, Bauer 2023). To maximize the ecological relevance, I measured the responses of a songbird species (black-capped chickadee, *Poecile atricapillus*; hereafter ‘chickadees’). Chickadees were exposed to the vocalizations of their natural predators (or non-predators) and were then housed in naturalistic flocks for 7 days post-exposure. Several

previous field manipulations using predator vocalizations have established that the fear of predators has population-level consequences in wild birds (Zanette et al. 2011, Dudeck et al. 2018, Allen et al. 2022). I followed the same protocol that I had previously used to experimentally demonstrate enduring, PTSD-like effects on neural activation and hypervigilance behaviour in this species (Zanette et al. 2019). My current results demonstrate that fear of predators has enduring effects on neurogenesis, with likely implications for memory.

2.2 Materials and methods

2.2.1 Study Species, Animal Housing and Predation Risk Manipulation

I used black-capped chickadees due to their abundance and year-round residency in Southern Ontario, and their ease in adapting to captivity. These same birds were used in a prior study to quantify the enduring effect of predator fear on Δ FosB in the brain (Zanette et al. 2019; see Discussion). Between September and November, twelve adult chickadees were captured using seed-baited Potter traps from sites around Western University, London, Ontario, Canada. Upon capture, chickadees were weighed, sex was estimated based on wing length (later confirmed with post-mortem examination of gonads), and chickadees were given a unique combination of colour bands for individual identification. Chickadees were captured at least seven days prior to the start of manipulations to acclimate to captivity and were housed in mixed sex groups in outdoor aviaries with ad libitum access to Mazuri small bird diet, black oil sunflower seeds, mealworms, and water.

Twelve chickadees were randomly assigned to either the predator or non-predator control treatment (Predator: 2F, 4M; Non-Predator: 4F, 2M). Individuals were relocated to cages within sound attenuating acoustic chambers 24 h prior to the manipulation. The chambers operated on a natural light cycle (11.5 h light: 12.5 h dark) with ad libitum access to food and water. Each chamber was outfitted with an MP3 player, speakers, and webcam for monitoring, and arranged so that the MP3 player and webcam could be operated without opening the chamber. Seven species were used in each treatment with species known to prey on chickadees selected for the predator treatment (Table 2.1),

matched to non-predator species for sound characteristics between groups (Zanette et al. 2019). All species selected for the playbacks occur locally and their vocalizations would all be heard naturally by chickadees in the area. All calls were obtained from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA) and the Xeno-Canto foundation (www.xeno-canto.org). Calls were broadcast at 74 dB SPL measured at the centre of the cage, with 5 minutes of calls playing at randomly selected intervals every hour. My manipulation ran for 48 h, with playbacks broadcast 12 h per day during daylight hours. Following playbacks individuals were returned to their flocks in the outdoor aviaries.

2.2.2 Brain Processing, Immunohistochemistry, and Image Analysis

Seven days following the playback chickadees were euthanized by overdose of isoflurane, then transcardially perfused with 0.1M phosphate buffered saline (PBS) (pH 7.4) and 4% paraformaldehyde. Brains were removed and placed in paraformaldehyde for 24 h, followed by 30% sucrose for 24 h until saturated and frozen at -80 °C. Brains were sectioned to 40 µm coronal slices on a cryostat, taking four series starting from the end of the anterior commissure until the end of the cerebral lobes. Each series consisted of every fourth slice collected. Slices were stored in cryoprotectant at -20 °C until immunohistochemistry began. I carried out immunohistochemistry on free-floating sections to label doublecortin (DCX (C-18) goat IgG, sc-8066, Santa Cruz Biotechnology) and proliferating cell nuclear antigen (PCNA (PC10) mouse IgG, sc-56, Santa Cruz Biotechnology) following established protocols (Diez et al. 2021), with the primary antibodies at a concentration of 1:250 and 1:1000 in 0.3% phosphate-buffered saline with triton (PBS/T), respectively. Sections were then labelled with a secondary antibody (horse anti-goat at 1:400 for DCX and goat anti-mouse at 1:250 for PCNA, Vector Laboratories) and visualized with diaminobenzidine solution.

For each marker, I quantified reactivity in the hippocampus (Hp; avian homologue to the mammalian hippocampus; Colombo and Broadbent 2000) medial ventral arcopallium (AMV; avian homologue to the amygdala; Yamamoto et al. 2005, Mello et al. 2019), and the caudolateral nidopallium (NCL; avian analogue to the

mammalian prefrontal cortex; Herold et al. 2011). For DCX all images were taken using a 40X objective lens to capture z-stack images (Figure 2.1). For the z-stacks, a series of photos were taken covering the depth of the tissue of interest, then optimized within the Leica Application Suite software to ensure that the entire field was in focus regardless of the depth. For the Hp, I took photos of the lateral, medial, and ventral subregions (Figure 2.1). For the AMV I took photos of the lateral and ventral subregions (Figure 2.1). Within each of the Hp, AMV, and NCL I took photos of each area of interest from each hemisphere on 5 slices. Images for the Hp were taken for the first five slices with both hemispheres present posterior to the anterior commissure. Images for the AMV and NCL were taken on slices where the region is present on both hemispheres starting from the anterior end of the AMV. Five slices were used for consistency with previous experiments quantifying DCX in the avian brain (Hall et al. 2014, Wada et al. 2014, Diez et al. 2021). I converted the images from colour to 16-bit greyscale, enhanced the contrast, then used the thresholding tool in ImageJ (Schneider et al. 2012) to convert the DCX positive cells and fibres to black against a white background, manually adjusted threshold to best match cells, and quantified the percent cover of DCX immunoreactivity. For PCNA, all images were taken using a 20X objective lens, with one photo taken from each hemisphere on each slice that the region of interest was present (Figure 2.2). This included eight slices from each of the Hp and NCL and six slices from the AMV. I then calibrated ImageJ (Schneider et al. 2012) to the image measurement, and measured the area of interest in mm^2 . I converted the images from colour to 16-bit greyscale, subtracted the background, and enhanced the contrast. I then used the thresholding tool to convert the PCNA positive nuclei to black cells on a white background, then used the count function to quantify the number of cells. Density was calculated as the PCNA positive cells/ mm^2 for each photograph. All images were collected and quantified without knowledge of the treatment groups or sex to avoid bias in the results.

2.2.3 Statistical Analysis

For each marker (i.e., DCX and PCNA) the mean value was calculated for each brain region within each individual animal. A two-way ANOVA was used to analyze the effects on each marker within each brain region measured, with treatment and sex as the

independent variables and the percent cover of DCX-immunoreactive cells or the density of PCNA-immunoreactive cells as the dependent variable. A Dunnett's post hoc test was used for PCNA, which compared non-predator males against the predator males, predator females, and non-predator females. When working with wild animals, it is necessary to balance the need to maximize my sample size with the need to minimize the number of animals euthanized. To support the above parametric analyses and minimize the potential for error due to sample size, a permutation test was conducted for all ANOVA results, based on 10000 permutations with the permuco package in R (Frossard and Renaud 2021). A permutation test is a non-parametric estimate of the population distribution that is used to estimate how rare the observed values are by sampling all possible permutations of the data without replacement and is robust and effective for small sample sizes. The Freedman-Lane permutation method was used due to it being the most accurate and powerful in controlling for type 1 errors (Winkler et al. 2014). All permutation results supported the parametric ANOVA results and are outlined in Table 2.2. All analyses were conducted in RStudio Version 1.4.1103.

2.3 Results

2.3.1 Enduring effects of fear on cell proliferation - PCNA

Exposure to predator playbacks one week earlier had an enduring effect on cell proliferation in the hippocampus (Hp) of males but not females (Figure 2.3A; 2-way ANOVA treatment x sex interaction: $F_{1,8}=6.89$, $p=0.03$; main effect on sex: $F_{1,8}=18.49$, $p=0.003$; main effect of treatment: $F_{1,8}=0.53$, $p=0.49$). Males in the non-predator control treatment displayed 43% more cell proliferation compared to their female counterparts (Dunnett's post hoc, $p=0.007$). However, 7 days after hearing predator playbacks, males decreased their cell proliferation by 30% ($p=0.045$) whereas females exhibited no change (Figure 2.3A). There were no statistically significant main effects or interactions for cell proliferation in the medial ventral arcopallium (AMV; Figure 3B; all $p>0.3$) or the caudolateral nidopallium (NCL; Figure 2.3C; all $p>0.2$)

2.3.2 Enduring effects of fear on immature neurons - DCX

Predator playback exposure also had enduring effects on immature neurons, but in this case, in the medial ventral arcopallium (AMV) and irrespective of sex. Chickadees who heard predator calls one week earlier showed a 16% reduction in immature neurons in the AMV (Figure 2.4B; $F_{1,8}=5.48$, $p=0.047$). Sex did not have a statistically significant main effect ($F_{1,8}=5.18$, $p=0.052$; means \pm SE, males versus females: 13.32 ± 1.03 versus 11.28 ± 1.08) and there was no treatment by sex interaction ($F_{1,8}=0.04$, $p=0.85$). I found no main effects or interaction of treatment and sex in the hippocampus (Hp; Figure 2.4A; all $p>0.2$) or the caudolateral nidopallium (NCL; Figure 2.4C; all $p>0.3$).

2.4 Discussion

My results demonstrate that a chronic perceived sense of heightened danger from predators leaves an enduring effect on neurogenesis. This is indicative of an enduring memory of fear, with some evidence of sex dependence, in brain regions associated with fear processing lasting at least one week after the threat has been removed. In the AMV (a proposed homolog of the mammalian amygdala; Yamamoto et al. 2005, Mello et al. 2019), immature neurons (DCX) were significantly suppressed for both males and females. In the Hp, immature neurons did not vary with treatment, but I did find an effect of treatment on cell proliferation (PCNA) according to sex. Males significantly reduced cell proliferation one week after hearing predators while females showed no change between treatments. I also found a sex difference in cell proliferation in the control treatment, suggesting that males and females may face different behavioural and ecological consequences from fear-induced alterations to hippocampal neurogenesis and highlighting the importance of including both sexes in any studies on neurobiology. Unlike the Hp and AMV, the NCL did not show an enduring effect of fear on either cell proliferation or immature neurons.

My results revealed that exposure to predator vocalizations reduced cell proliferation, defined here as PCNA-ir cells, in males, while females showed no statistically significant difference. PCNA has been widely used to label proliferation of new neurons in the avian brain (Mazengenya et al. 2017, 2018, 2020, Diez et al. 2021),

however it can also be found in non-neuronal dividing cells (Wang 2014). It is widely accepted that adult neurogenesis occurs in the hippocampus (Christie and Cameron 2006), with cell proliferation in the songbird brain occurring in the subventricular zone (Pytte 2016). The cells measured here are likely very young cells that have very recently migrated away from the subventricular zone while PCNA is still present in the cell. Given the 20 h half-life of PCNA in rodents (Bravo and Macdonald-Bravo 1987), this suggests both that fear is directly impacting the rate of hippocampal neurogenesis and that this effect endures long after the threat has passed. Indeed, this same pattern has been demonstrated in rats in the lab in which exposure to fox odour led to a decrease in cell proliferation 24 hours later in males with no effect in females (Falconer and Galea 2003). Similarly, a suppression in cell proliferation was also documented in the forebrain of fish living in a naturally high predator environment compared to a population experiencing naturally low predation pressure with no difference reported between sexes (Dunlap et al. 2016).

Cell proliferation in the Hp also differed between the sexes in my non-predator control treatment, whereby males had 43% more PCNA immunoreactive cells than females, which is consistent with previously reported sex differences in the ventricular zone of the avian brain (Katz et al. 2008). In winter flocks, chickadees form stable dominance hierarchies with males generally dominant over females (Smith 1991). Dominant chickadees have been shown to have more newly proliferated cells in the ventricular layer of the Hp than do subordinates (Pravosudov and Omanska 2005), which may explain my results in the control group. If reduced cell proliferation enhances memory retention as previous studies have indicated (Frankland et al. 2013, Akers et al. 2014), then it is possible that by suppressing hippocampal cell proliferation in response to predators male chickadees make a trade-off that prioritizes memory of predators at the expense of maintaining dominance status. Dominant males and their female mates typically access the highest quality breeding sites during the following breeding season (Smith 1991). However, even a lower quality breeding site would still give a greater potential fitness benefit than being killed by a predator before the breeding season begins. Further behavioural studies would be needed to better elucidate the ecological consequences my reported sex differences in hippocampal cell proliferation. This

highlights the importance of including both sexes, as the historic trend to favour males in lab animal models for PTSD (Deslauriers et al. 2018) may lead to overgeneralization or important interactions being overlooked due to a lack of female subjects.

In the AMV (proposed homolog of the mammalian amygdala; Yamamoto et al. 2005, Mello et al. 2019), I found a reduction in immature (DCX+) neurons in both sexes one week after the predator treatment. While the AMV and its mammalian counterpart the amygdala are not historically known as key neurogenic regions, there is evidence for neurogenesis occurring in the mammalian amygdala (Fowler et al. 2008, Jiang et al. 2014, Jhaveri et al. 2018, Jurkowski et al. 2020, Sánchez-Gomar et al. 2024). In the avian brain, adult neurogenesis is widespread (Brenowitz and Larson 2015), suggesting that neurogenesis outside of the hippocampus is much more common in birds than mammals. While quantifying DCX immunoreactivity with percent cover does include both the migrating fusiform cells and the recently differentiated multipolar cells (Balthazart and Ball 2014), fusiform cell counts have been shown to positively correlate to percent cover of DCX-ir cells in regions associated with avian vocal control and auditory perception (Aitken et al. 2024), suggesting that treatment effects on percent cover of DCX-ir cells capture differences in newly formed, migrating cells. Given that I found enduring effects in the AMV only on immature neurons (DCX), with no corresponding effect on cell proliferation (PCNA), it is also possible that these immature neurons migrated from the ventricular zone at a later stage of the cell cycle, because doublecortin expression can be measured in cells for at least 60 days from formation (Balthazart and Ball 2014, Vellema et al. 2014) and has been shown to correlate to neuronal migration (Hannan et al. 1999). Neuronal migration may also explain the lack of a statistically significant enduring effect of fear on immature neurons in the Hp, while males showed a significant reduction in hippocampal cell proliferation, particularly if predator induced fear plays a role in the rate at which these newly proliferated neurons are migrating from the Hp to other areas of the brain.

My results suggest that exposure to predator cues suppressed neurogenesis, as indicated by both the immature neurons of the AMV of males and females and in cell proliferation in the Hp of males. Reduced hippocampal neurogenesis has been

demonstrated to increase memory persistence (Akers et al. 2014), while increased neurogenesis weakened trauma related memories and attenuated PTSD-like behaviours in lab mice (Fujikawa et al. 2024). Reduced neurogenesis has also been shown to impede reference memory reversal in chickadees (Guitar and Sherry 2018), supporting the idea that suppression of neurogenesis helps prevent the replacement of existing memories (Frankland et al. 2013). While the Hp is most often associated with memory formation, particularly in the avian brain (Sherry and Hoshooley 2007), the amygdala has also been found to play an essential role in the consolidation, storage, and recall of ‘emotional’ memories, particularly those memories provoked by fear (Richter-Levin 2004, Hermans et al. 2014, Diamond and Zoladz 2016, Hammack et al. 2023b, 2023a). Fos activation in the amygdala has also been used as a marker of remote memory recall (Hammack et al. 2023b, 2023a), and is consistent with increased Δ FosB activation and increased hypervigilance behaviour previously reported for these same chickadees under this same experimental protocol (Zanette et al. 2019). Interestingly, in a previous fear manipulation I conducted on wild birds (brown-headed cowbirds) in semi-natural conditions in the field, I also documented suppressed DCX immunoreactivity in the AMV one week after predator exposure (Witterick et al. unpublished results). For humans with PTSD, it is a hyperfunctional amygdala that is thought to maximize individual survival through heightened vigilance to minimize the chance of a surprise predator encounter, an evolutionarily primitive mechanism to maximize survival (Diamond and Zoladz 2016). This suggests that the enduring suppression of immature neurons seen in the AMV is contributing to an enduring memory of predator-induced fear. Sex differences in rates of cell proliferation without corresponding sex effects on immature neurons have previously been attributed to differences in cell survival (Galea et al. 2006), which may be a factor in why I saw suppression of hippocampal cell proliferation only in males but suppression of immature neurons in the AMV in both sexes. Further research quantifying apoptosis along with neurogenesis could be an interesting next step to understanding how much of the reduction in immature neurons is caused by a reduction in new cells being produced compared to how many cells are undergo apoptosis before reaching maturity. Given that cell proliferation converges between the sexes in the predator treatment and there is no sex difference in the suppression of immature neurons, it is possible that the sex

difference represents two different regulatory pathways to a hyperfunctional amygdala retaining an enduring memory of fear.

Enduring suppression of neurogenesis indicative of an enduring memory of fear, particularly in wild caught animals who likely already had a baseline level of previous predator experience, supports the notion that there may be an evolutionarily adaptive advantage to PTSD if the memory of that traumatic event is advantageous for survival (Cohen et al. 2023). Retaining an enduring memory of fear may also have evolutionary advantages enduring past the lifetime of the individual, as adult mice who experienced a 5 minute predator encounter produced offspring and grand-offspring who showed enduring anxiety-like behaviour and hyperarousal measured in response to mild stressors (2 minute predator exposure), in addition to increased hippocampal activation in the offspring (Bhattacharya et al. 2023). Intergenerational transmission of PTSD has also been documented in humans, although further research is needed to elucidate the mechanisms behind this (reviewed in Bowers and Yehuda 2016, Yehuda and Lehrner 2018). To further understand these potential adaptive advantages, the next step would be to investigate whether comparable enduring effects can be seen in wild animals outside of the controlled laboratory environment, with predator exposure occurring either under semi-natural conditions in captivity or in free-living wildlife. Finding PTSD-like effects indicative of an enduring memory of fear in a wild caught animal suggests that while the costs of a predator encounter may be far greater than the opportunity costs of reduced foraging as seen in the ecology of fear (Brown et al. 1999, Brown 2019), these enduring effects of fear are likely common in nature and may give an evolutionarily adaptive advantage.

Table 2.1: List of species used in the auditory playbacks for the chickadees. Predator and non-predator species were matched based on their acoustic call characteristics (frequency and maximum amplitude).

Predators	Non-Predators
Cooper's hawk, (<i>Accipiter cooperii</i>)	Song sparrow (<i>Melospiza melodia</i>)
American crow (<i>Corvus brachyrhynchos</i>)	Mallard (<i>Anas platyrhynchos</i>)
Red-tailed hawk (<i>Buteo jamaicensis</i>)	Blue jay (<i>Cyanocitta cristata</i>)
Barred owl (<i>Strix varia</i>)	Northern leopard frog (<i>Lithobates pipiens</i>)
Sharp-shinned hawk (<i>Accipiter striatus</i>)	Hairy woodpecker (<i>Picoides villosus</i>)
Northern saw-whet owl (<i>Aegolius acadicus</i>)	Wood frog (<i>Lithobates sylvaticus</i>)
Merlin (<i>Falco columbarius</i>)	Downy woodpecker (<i>Picoides pubescens</i>)

Table 2.2: Statistical results for the parametric ANOVA and the permutation ANOVA.

Marker	Brain Region	Factor	Parametric F	Parametric p	Permuted p
PCNA	Hp	Treatment	0.53	0.49	0.15
		Sex	18.49	0.003	0.01
		Treatment*Sex	6.89	0.030	0.026
	AMV	Treatment	0.67	0.44	0.85
		Sex	0.34	0.57	0.88
		Treatment*Sex	0.92	0.36	0.37
	NCL	Treatment	1.41	0.27	0.35
		Sex	0.20	0.66	0.96
		Treatment*Sex	0.47	0.51	0.52
DCX	Hp	Treatment	0.75	0.41	0.88
		Sex	0.24	0.64	0.51
		Treatment*Sex	1.91	0.20	0.21
	AMV	Treatment	5.49	0.047	0.042
		Sex	5.18	0.052	0.051
		Treatment*Sex	0.04	0.85	0.87
	NCL	Treatment	0.02	0.88	0.50
		Sex	0.62	0.45	0.85
		Treatment*Sex	0.85	0.38	0.37

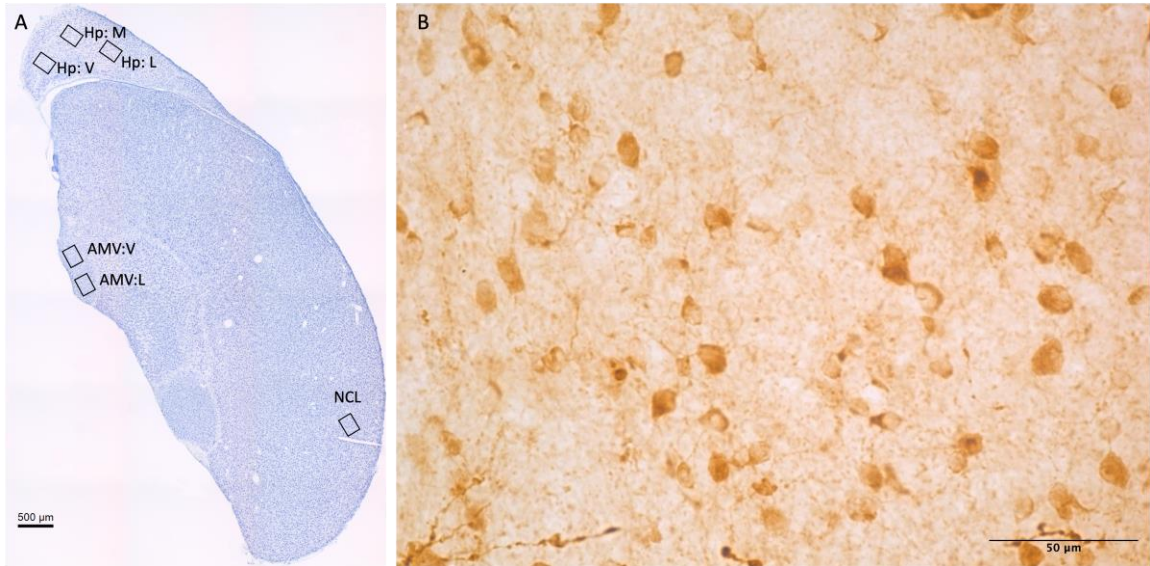


Figure 2.1: A) Photomicrograph of a Nissl stained coronal section of chickadee telencephalon. Boxes depict locations where images were taken for DCX quantification within each brain region measured (L: lateral, M: medial, V: ventral). B) Higher magnification photomicrograph of DCX immunoreactivity in the lateral AMV.

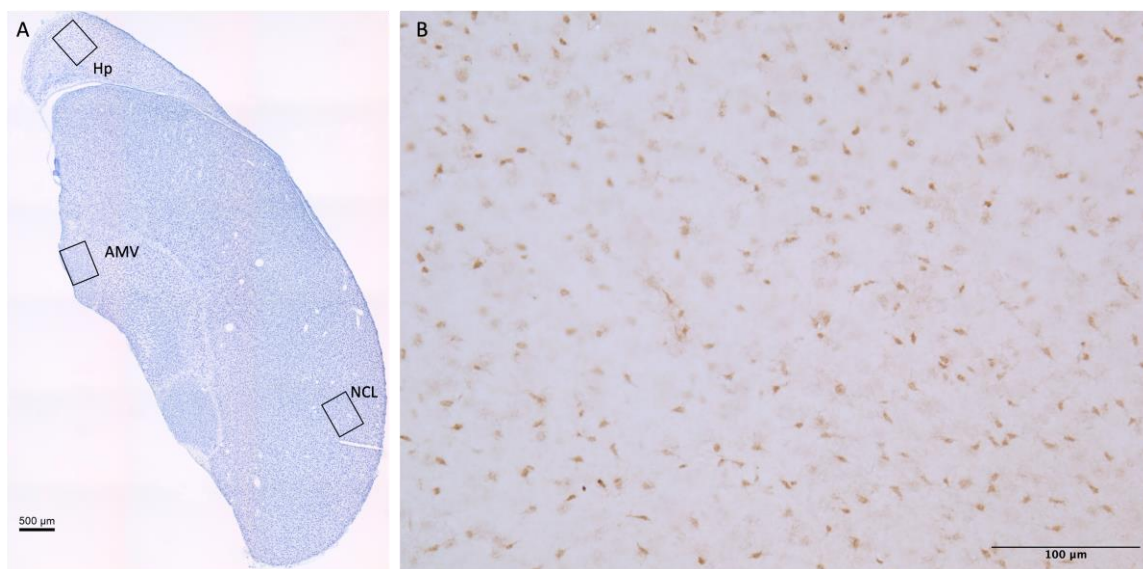


Figure 2.2: A) Photomicrograph of a Nissl stained coronal section of chickadee telencephalon. Boxes depict locations where images were taken for PCNA quantification. B) Higher magnification photomicrograph of PCNA immunoreactivity taken in the Hp.

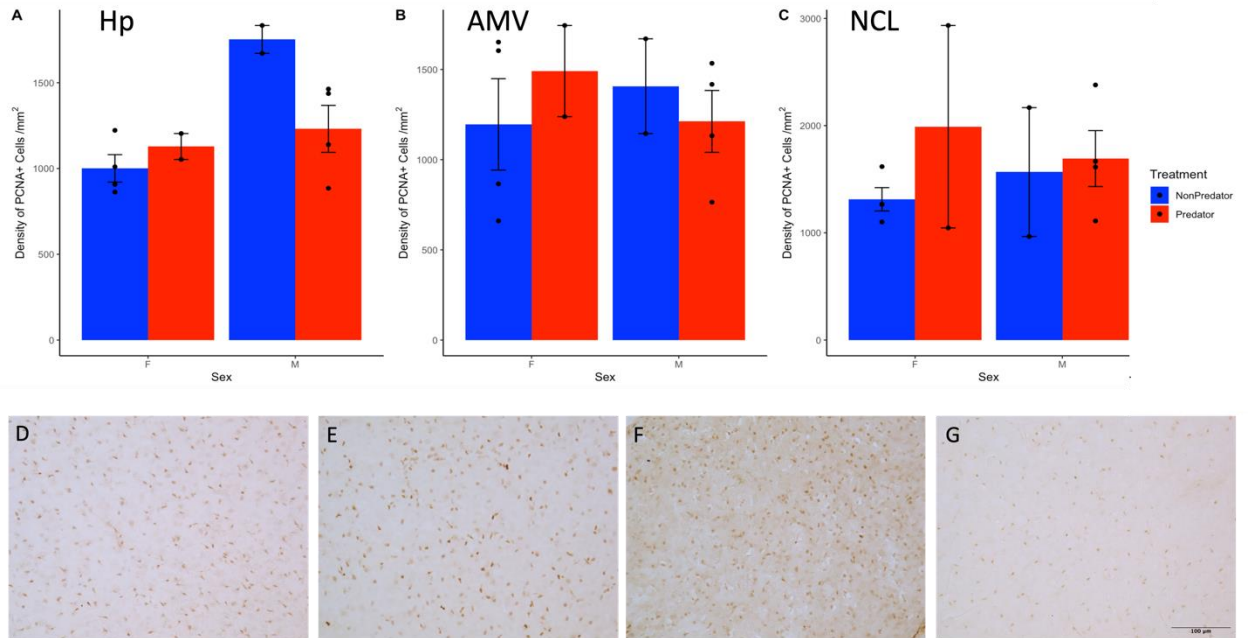


Figure 2.3: Cell Proliferation: The effects of predator induced fear on the PCNA+ cell density in the A) hippocampus (Hp; treatment x sex $p=0.03$), B) medial ventral arcopallium (AMV; treatment x sex $p=0.4$), and C) the caudolateral nidopallium (NCL; treatment x sex $p=0.5$). Bars indicate means \pm standard error, filled circles indicate individual data. Example photomicrographs of PCNA immunoreactivity in the Hp of a D) non-predator female, E) predator female, F) non-Predator male, and G) predator male.

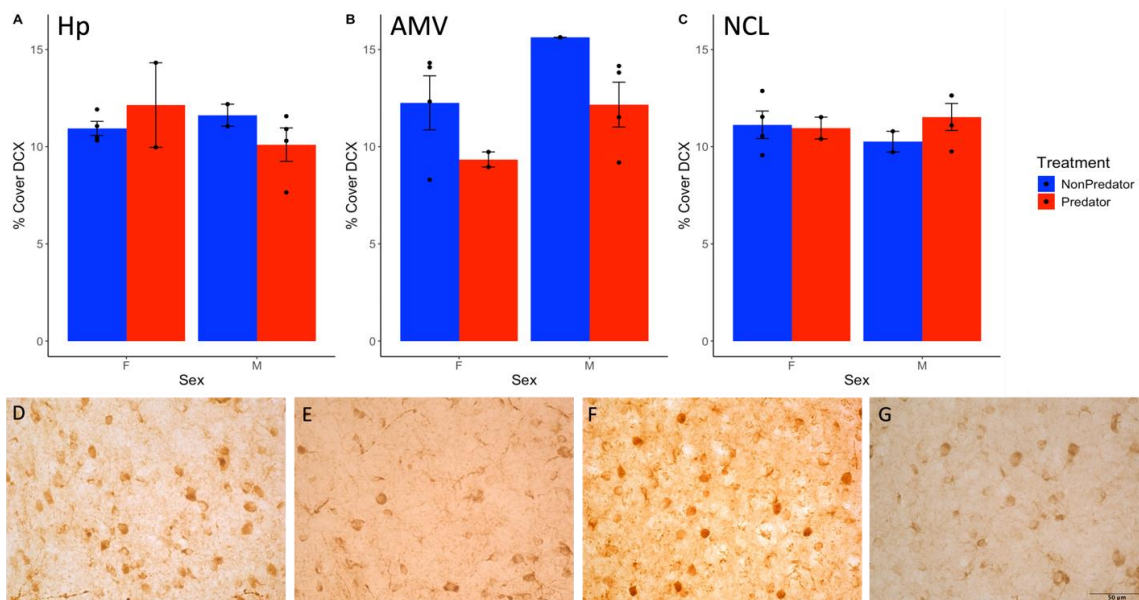


Figure 2.4: Immature Neurons: The effects of predator-induced fear on the percent cover of DCX+ cells in the A) hippocampus (Hp; treatment $p=0.4$), B) medial ventral arcopallium (AMV; treatment $p=0.047$), and C) the caudolateral nidopallium (NCL; treatment $p=0.9$). Bars indicate means \pm standard error, filled circles indicate individual data. Example photomicrographs of DCX immunoreactivity in the AMV of a D) non-predator female, E) predator female, F) non-Predator male, and G) predator male.

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Chapter 3

3 Fear of predators alters neuron structure in the brains of wild songbirds

3.1 Introduction

Predators scare prey, which can have immediate, transient effects on the animal, in addition to behavioural changes with enduring consequences for the individual, population, and community as characterized by the ecology of fear (Brown et al. 1999, Brown 2019, Zanette and Clinchy 2019, 2020). Laboratory studies demonstrating enduring effects of fear on neurobiology suggest that wildlife should see changes in the brain occurring concurrently with the behavioural changes in response to predator-induced fear, however little is known about the enduring effects of fear on the brain in wildlife living outside of the laboratory confines. Two previous studies have shown enduring effects of fear on the brain and behaviour in wild caught black-capped chickadees in the laboratory (Zanette et al. 2019, Chapter 2), raising questions as to whether comparable enduring effects of fear on the brain could be demonstrated amid the environmental variation and additional associated challenges wild animals experience outside of the controlled laboratory environment.

To quantify the enduring effects of fear on the brain I can use tools from animal models for post-traumatic stress disorder (PTSD), where live predators or their cues (sounds, odour, or mounts) are frequently used to induce enduring, PTSD-like behaviours in lab animal models (Deslauriers et al. 2018). In small bodied animals such as rodents or birds, animal models require that effects persist for at least 7 days to be considered an enduring, PTSD-like effect, rather than just an immediate, transient response to predation risk (Deslauriers et al. 2018). While laboratory models provide us with a wealth of valuable research on the enduring effects of fear on captive animals, the question has also been raised as to whether effects seen in the laboratory would translate to their wild counterparts (Clinchy et al. 2011, Cohen et al. 2023). Restraint stress is another commonly used method to induce PTSD-like symptoms and conventional laboratory housing often restricts natural behaviours due to a lack of available refuges and

opportunities for nesting or burrowing (Richter-Levin et al. 2019, Cait et al. 2022), and may be perceived by the animals as a mild form of restraint. Enriched housing has been demonstrated to improve health outcomes, increase lifespans, and mitigate anxiety-like behaviour (Cait et al. 2022), suggesting that there may be a potential interaction between housing conditions and the behavioural and neural response to predator-induced fear. Manipulating predation risk under semi-natural conditions, where animals are physically protected from predators but remain exposed to their natural environment and have the space to perform their natural anti-predator behaviours such as seeking refuge, minimizes the potential interactions with captivity and can expand upon and validate any comparable PTSD-like findings measured in a laboratory environment (Clinchy et al. 2011, 2013).

Laboratory animal models also provide us with the tools to quantify the enduring effects of fear on the brain through changes to neuron structure, activation, and neurogenesis. Enduring effects of fear can be measured through changes to neuron structure, often measured through changes in dendritic length and branching. Alterations to dendritic morphology in response to predators or their cues have been demonstrated in the hippocampus (Hoffman et al. 2016, Cohen et al. 2020, 2023) and basolateral amygdala (Mitra et al. 2009, Adamec et al. 2012), while comparable effects have been demonstrated in the prefrontal cortex in response to restraint stress (Liston et al. 2006, Radley et al. 2006, Garrett and Wellman 2009) in laboratory rodents. Neurogenesis can be assessed at various stages, including both cell proliferation using proliferating cell nuclear antigen (PCNA) and immature neurons with doublecortin (DCX). The suppression of neurogenesis has been demonstrated to promote memory persistence by preventing the replacement of existing memories (Frankland et al. 2013, Akers et al. 2014). After chronic stress, mice experiencing experimentally suppressed neurogenesis took longer to recover from anxiety like behaviours, suggesting that suppressed neurogenesis has an enduring, PTSD-like effect on memory (Fujikawa et al. 2024). One week after hearing predator calls, I found suppressed cell proliferation in the hippocampus (Hp) in males and fewer immature neurons in the medial ventral arcopallium (AMV; avian homologue to the amygdala; Yamamoto et al. 2005, Mello et al. 2019) in both sexes of chickadees in my previous laboratory experiment (Chapter 2).

Activation in this thesis is defined as an enduring marker of earlier immediate early gene expression which can be quantified using Δ FosB, a uniquely stable member of the FOS family of immediate early gene transcription factors, with a half-life of up to 8 days (Robison and Nestler 2022). While widely studied for its role in addiction, induction of hippocampal Δ FosB has also been linked to stress resilience, learning, and memory (Robison and Nestler 2022). Enduring increases in Δ FosB have been reported in both rodents (Staples et al. 2009, Mackenzie et al. 2010) and chickadees in the laboratory (Zanette et al. 2019).

In this experiment, I exposed flocks of black-capped chickadees (*Poecile atricapillus*; hereafter ‘chickadees’) to predator calls under semi-natural conditions in large outdoor aviaries, allowing animals to experience natural environmental variability while still being protected from actual predation. I built upon previous laboratory experiments quantifying the enduring effects of predator-induced fear on the brain and behaviour in chickadees (Zanette et al. 2019, Chapter 2), taking chickadees outdoors to semi-natural conditions (following Witterick 2017). Chickadees were housed in group flocks and allowed to form a social dominance hierarchy, matching the social groups seen in their wild counterparts during the fall and winter when this experiment was conducted (Smith 1991). Following the ecology of fear, I looked at the effects of predation on foraging behaviour, as reduced foraging in response to predators can have individual, population, and community level consequences. Building on previous laboratory studies in captivity, I quantified the enduring effects of the fear of predators on activation, cell proliferation, immature neurons, and dendritic morphology in three brain regions (the avian counterparts to the mammalian hippocampus, prefrontal cortex, and amygdala) commonly associated with fear processing.

3.2 Methods

3.2.1 Study Site and Animal Housing

Between September and November 2019, 47 black-capped chickadees (*Poecile atricapillus*; 17F, 30M) were captured using seed baited Potter traps from multiple sites around London, Ontario, Canada. Upon capture, chickadees were weighed, sex was

estimated based on wing length (later confirmed with post-mortem examination of gonads) and each bird was given a unique combination of colour bands for individual identification. Chickadees were housed in mixed sex groups of six in outdoor aviaries at the Advanced Facility for Avian Research (AFAR), Western University with *ad libitum* access to Mazuri small bird diet, black oil sunflower seeds, mealworms, and water. The first 24 chickadees were then transported to the Environmental Sciences Western (ESW) field station, with 23 chickadees remaining at AFAR to remain isolated from the treatments for the second round of the experiment. At ESW, six birds were housed in each of four large outdoor aviaries (3.66 m x 9.14 m x 18.29 m). Each aviary was equipped with perches, trees, grass, an A-frame shelter, and two nesting boxes, providing a semi-natural environment while protecting birds from predation. Adjacent aviaries were separated by an opaque barrier, visually isolating the groups. The aviaries pairs were separated by 150 m, separating groups both visually and acoustically. Chickadees had *ad libitum* access to Mazuri small bird diet, black oil sunflower seeds, and water, in addition to foraging for any naturally present food in the aviaries.

3.2.2 Predation Risk Manipulation

To manipulate perceived predation risk, chickadees were exposed to auditory playbacks of predator or non-predator species (Table 3.1) between 4 November and 21 December 2019. Chickadees were given a minimum of one week in the large outdoor aviaries to acclimate and form a flock hierarchy before the playbacks began. Following Witterick (2017), I conducted two rounds of the experiment, where each treatment was conducted in each of the aviary pairs, to account for any differences in the micro-climate or surrounding environment between the aviary locations. Each experimental round consisted of 10 days of playbacks on a 4 days on, 2 days off, 4 days on schedule to avoid habituation, followed by seven days with no audio to quantify the enduring effects on the brain (Figure 3.1). To prevent sound contamination, adjacent aviaries received the same treatments simultaneously, with the schedule staggered by three days between aviary pairs to allow for behavioural observations to be conducted on the same experimental day for each treatment.

Chickadees were randomly assigned to aviary groups and treatments while maintaining a balanced sex ratio across treatments. For the predation risk manipulation, each aviary was equipped with two playback units housed in weatherproof boxes (containing a pair of speakers) and MP3 player. Playback units were mounted 2.4 m high, at least 12 m apart from each other and moved to a new location within the aviary every two days to prevent habituation. Seven different species were used for each playback treatment, matched to ecologically relevant time of day (Table 3.1). Sounds of predator or non-predator species were broadcast at 80 dB SPL from 1 m away with a call-to-silence time ratio of 1:1.5 during the day and 1:2.3 during the night to simulate temporal variations in predation risk and randomized so that only one unit was broadcasting within each aviary at any given time.

3.2.3 Behaviour Assays

To assess foraging behaviour, feeding platforms were placed in each of the aviaries 1 m distance from the protective cover of cedar trees. Trials were conducted the day before playbacks began, on Day 1 of playbacks, and 6 days after the playbacks finished (1 day before brains were collected). On each trial day, the feeders were removed from the aviaries between the hours of 9:00 and 15:00 and replaced with a block containing ~95 g of lard with 50 g of black oil sunflower seeds on each platform. Feeding platforms were recorded with a waterproof camera for 2 h in either the morning or afternoon to assess foraging behaviour (balanced between replicates of each treatment, i.e. if the north side of the aviary was filmed in the morning of Day 1 for round one it was filmed in the afternoon of Day 1 in round two). Videos were then scored in Solomon Coder (A. Peter, <https://solomon-coder.software.informer.com/>) to quantify the frequency of visits to feeding platform along with the total time feeding, vigilance, and movement for each aviary group (see Table 3.2 for definitions). All videos were scored blind to the aviary treatments. The proportion of time on camera that a behaviour was being performed was then calculated to standardize the behavioural data for any variation in the video length and the total number of visits to the foraging platforms per hour was calculated for each aviary group.

Chickadees were also assessed for dominance status, as dominance status has been linked to risk taking behaviours (Fox et al. 2009, An et al. 2011). To determine the social hierarchy, interactions were recorded on a feeding platform on experimental days 11-13 (days 1-3 post treatment). All food was removed from the aviary for 1.5 h, after which black oil sunflower seeds were placed on the closest feeding platform (1 m from protective cover) along with a waterproof camera to record interactions between individuals in the flock for 60 min. Videos were scored in Solomon Coder to quantify interactions such as individuals being chased, resisted, submissive, or waiting (see Table 3.2 for definitions). The interactions were then summed in a dominance interaction matrix and the EloRating package in R was used to calculate the hierarchy based on the normalized David's Score (de Vries et al. 2006).

3.2.4 Brain Processing

Seven days after the playbacks ended, brains were collected for the neurobiology assays. Chickadees from each aviary were randomly assigned to be processed for immunohistochemistry (9F and 14M) or dendritic morphology (8F and 16M), while splitting each sex between treatments for each measure. I recaptured chickadees with seed baited potter traps and euthanized by isoflurane overdose. For chickadees assigned to dendritic morphology, brains were removed immediately following euthanasia and incubated in Golgi-Cox Solution (1% potassium dichromate, 0.8% potassium chromate, and 1% mercuric chloride in water) in the dark for 25 days, then transferred to 30% sucrose for 48 h until saturated and frozen at -80 °C for long term storage. Chickadees assigned to immunohistochemistry were transcardially perfused with 0.1M phosphate buffered saline (PBS) (pH 7.4) and 4% paraformaldehyde. Brains were removed and left in paraformaldehyde for a minimum of 7 days to ensure fixation, followed by 30% sucrose for 48 h until saturated, and then frozen at -80 °C for long term storage.

3.2.5 Golgi Staining and Neuron Tracing

Golgi-Cox stained brains (NP: 5F, 7M; Pred: 3F, 9M) were sliced to 500 µm coronal slices using a vibratome. Brains were sliced from the posterior to the anterior, collecting slices from the posterior of the brain to the end of the anterior commissure. Brains were

processed following Louth et al. (2017). Brains were incubated overnight in 6% sucrose solution at 4 °C, then developed in 2% paraformaldehyde, followed by 2.7% ammonium hydroxide, and then 10% Kodak Fixative A, with slices washed in distilled water between each step. All imaging was done using NeuroLucida, with image stacks taken with a 30X objective lens for 4 pyramidal neurons in each of the Hp, AMV, and NCL from each individual (Figure 3.2). Neurons were selected from the two slices where the region of interest was present, with one neuron selected from each hemisphere of that slice (4 neurons per brain region, 12 neurons total from each individual). To be selected, neurons had to be fully visible within the slice of interest and not obscured by any other neuron or blood vessel. Each image stack took a photo at 1µm intervals to photograph the entire neuron, ranging from 50-150 photos depending on the size of the individual neuron. If the neuron exceeded the frame of view in the x or y plane, a second photo stack was taken and stitched together in ImageJ (Schneider et al. 2012) prior to tracing. Neurons were traced using Neuromatic (Myatt et al. 2012), with reconstructions converted back to NeuroLucida files using Neuronland. Data was then exported from NeuroLucida and converted from pixels to µm for analysis.

3.2.6 Immunohistochemistry and Image Analysis

A cryostat at -20 °C was used to section brains (NP: 5F, 7M; Pred: 4F, 7M) into 40 µm coronal slices for immunohistochemistry. I collected four series, with each series collecting every fourth slice in phosphate buffered saline. I began collecting sections when the tractus septopallio-mesencephalicus splits and collected until the end of the cerebral lobes. I carried out immunohistochemistry on free-floating sections to label ΔFosB (FosB (102) rabbit IgG, sc-48, Santa Cruz Biotechnology), doublecortin (DCX (C-18) goat IgG, sc-8066, Santa Cruz Biotechnology), and proliferating cell nuclear antigen (PCNA (PC10) mouse IgG, sc-56, Santa Cruz Biotechnology) following established protocols (Zanette et al. 2019, Diez et al. 2021), with the primary antibodies at a concentration of 1:500 (ΔFosB), 1:250 (DCX), and 1:1000 (PCNA) in 0.3% phosphate-buffered saline with triton (PBS/T), respectively. Sections were then labelled with a secondary antibody (goat anti-rabbit at 1:250 for ΔFosB, horse anti-goat at 1:400

for DCX, and goat anti-mouse at 1:250 for PCNA, Vector Laboratories) and visualized with diaminobenzidine solution.

For each marker, I quantified immunoreactivity in the hippocampus (Hp; avian homologue to the mammalian hippocampus; Colombo and Broadbent 2000), medial ventral arcopallium (AMV; avian homologue to the mammalian amygdala; Yamamoto et al. 2005, Mello et al. 2019), and the caudolateral nidopallium (NCL; avian analogue to the mammalian prefrontal cortex; Herold et al. 2011). For Δ FosB, all images were taken with a 10X objective lens (Figure 3.3) and for PCNA, all images were taken with a 20X objective lens (Figure 3.4). Images for the Hp were taken from starting from the slice posterior to the anterior commissure until the end of the hippocampus (8 slices with one image per hemisphere). For the AMV, images were captured for all slices the region was present (5 slices with one image per hemisphere). Images for the NCL were captured for 8 slices starting from the beginning of the AMV. For every slice photographed I captured a z-stack image of the region of interest for each hemisphere. The z-stack took images covering the depth of the slice of interest, then the photos were combined in the Leica Application Suite software to produce a single image with the entire field in sharp focus regardless of the depth within the slice. I then calibrated ImageJ (Schneider et al. 2012) to the image measurement, and measured the area of interest in mm^2 . I converted the images from colour to 16-bit greyscale, subtracted the background, and enhanced the contrast. I then used the thresholding tool in ImageJ to convert the Δ FosB or PCNA immunoreactive nuclei to black cells on a white background, then used the count function to quantify the number of cells in the area of interest. Density was calculated as the Δ FosB or PCNA immunoreactive cells/ mm^2 for each photograph. For DCX, all images were taken at with a 40X objective lens (Figure 3.5). For the Hp, I took photos at the lateral, medial, and ventral subregions. For the AMV I took photos of the lateral and ventral subregions. Within each of the Hp, AMV, and NCL I took photos of each area of interest from each hemisphere on 5 slices. Images for the Hp were taken for the first five slices with both hemispheres present posterior to the anterior commissure. Images for the AMV and NCL were taken on slices where the region is present on both hemispheres starting from the anterior end of the AMV. I converted the images from colour to 16-bit greyscale and enhanced the contrast. I then used the thresholding tool in ImageJ to

convert the DCX positive cells and fibres to black against a white background and quantified the percent cover of DCX positive cells. All images were collected and quantified without knowledge of the treatment groups or sex to avoid bias in the results.

3.2.7 Statistical Analysis

The foraging behaviour assay was analyzed using an ANOVA at the aviary level, with visits to the foraging platform per hour as the dependent factor and treatment independent variable, and repeated for before, during, and after playback. Effects were compared separately for each time point (before, during, and after playbacks) with a Bonferroni correction to adjust p -values to account for multiple comparisons.

For dendritic morphology, the average length and number of branch nodes were calculated to give one value per brain region per individual for each measure. These were then standardized to a z -score for each region and zeroed around experimental round to control for any differences between replicates. The z -scored data was then analyzed with a two factor ANOVA, with treatment and sex as fixed factors.

For the immunohistochemistry assays, the average of each marker was calculated for each brain region, to give one value per brain region per individual for each of Δ FosB, DCX, and PCNA. These were analyzed using a three factor ANOVA, with treatment, sex, and experimental round as fixed factors. When experimental round was not a significant factor in the analysis, aviary rank was included as a covariate to account for dominance rank within the aviary. Experimental round and the dominance status covariate were not significant effects in any of the models, therefore they will not be reported in the results. All analyses were conducted in R Version 1.4.1103.

3.3 Results

3.3.1 Foraging Behaviour

Chickadees foraged at similar rates before playbacks began (Figure 3.6; $p=0.25$), but significantly reduced their feeding visits per hour by almost 2-fold (Figure 3.6; $p=0.048$) when exposed to predator playbacks. Chickadees who heard predator playbacks one week

earlier made slightly fewer visits after playbacks ended, this was not a statistically significant enduring effect (Figure 3.6; $p=0.432$).

3.3.2 Dendritic Morphology

Enduring effects were evident in dendritic morphology in key brain regions associated with fear processing. Chickadees that heard predator vocalizations one week previously had longer dendrites in both the Hp (Figure 3.7A; $F_{1,20}=4.49$, $p=0.047$) and the NCL (Figure 3.7B; $F_{1,20}=8.45$, $p=0.0087$), the latter of which also demonstrated a significant increase in dendritic branching (Figure 3.7E; $F_{1,20}=5.42$, $p=0.03$). No enduring effect of treatment was demonstrated in the AMV for dendritic length or branching (Figure 3.7C, 3.7F; all $p>0.1$), nor was there an effect on dendritic branching in the Hp (Figure 3.7D; $p>0.1$). There was no main effect or interaction with sex for dendritic length or branching in any brain region measured (all $p>0.1$).

3.3.3 Neurogenesis

Cell proliferation, as measured by PCNA immunoreactivity, was not affected by the fear of predators in the Hp, NCL, or AMV (Figure 3.8; Hp: Predator 1083.3 ± 54.1 , Non-predator 1050.3 ± 82.2 ; NCL: Predator 1432.1 ± 84.1 , Non-predator 1377.0 ± 109.9 ; AMV: Predator 2296.5 ± 144.1 , Non-predator 2280.8 ± 171.1 ; mean PCNA-ir cells/mm² \pm STE, all $p>0.1$). I also detected no effect of fear of predators on immature neurons, as measured through DCX immunoreactivity, in the Hp, NCL, or AMV (Figure 3.9; Hp: Predator 9.4 ± 0.47 , Non-predator 9.8 ± 0.50 ; NCL: Predator 10.6 ± 0.48 , Non-predator 11.1 ± 0.35 ; AMV: Predator 9.7 ± 0.40 , Non-predator 9.6 ± 0.35 ; all percent cover DCX-ir cells, all $p>0.1$).

3.3.4 Activation

Fear of predators had no significant effect on activation, measured by Δ FosB immunoreactivity, in the Hp, NCL, or AMV (Figure 3.10). There were no main effects or interactions with treatment or sex (Hp: Predator 543.0 ± 47.1 , Non-predator 540.9 ± 42.6 ; NCL: Predator 815.3 ± 65.3 , Non-predator 840.3 ± 74.9 ; AMV: Predator 635.9 ± 42.3 , Non-predator 676.7 ± 53.2 ; mean Δ FosB-ir cells/mm² \pm STE, all $p>0.1$).

3.4 Discussion

My results demonstrate that hearing predator vocalizations under semi-natural conditions alters prey foraging behaviour with enduring effects on dendritic morphology in the hippocampus (Hp) and caudolateral nidopallium (NCL; avian analogue to the mammalian prefrontal cortex; Herold et al. 2011). I found that when hearing playbacks of predator vocalizations, birds made fewer visits to feeding platforms. I also demonstrate that one week after hearing predator calls, chickadees had longer neurons in both the Hp and NCL, along with more branch nodes in the NCL when compared to the non-predator controls. I found no enduring effects of fear on neurogenesis or activation for any of the brain regions measured in this study.

Chickadees altered their behaviour when hearing calls of their predators, making fewer visits per hour to the feeding platforms compared to the non-predator controls. This is consistent with experimental models suggesting that prey alter their behaviour to minimize potential exposure to predators (Lima and Dill 1990) and with the ecology of fear, where increased risk of predation leads animals to reduce foraging, with population level consequences (Brown et al. 1999, Zanette and Clinchy 2020). I established that no difference between treatments was present before playbacks started, suggesting that there were no pre-existing behavioural differences or environmental variation between treatment groups or aviary locations. I also reported no enduring effect of treatment on foraging behaviour after playbacks, with a downward trend in the overall feeding visits over the course of the experiment. Given that chickadees are known to cache food and later return to retrieve food caches (Sherry 1984), it is possible that food was cached during periods of lower perceived risk, such as during the 5 days between the end of playbacks and the final day of observations, and therefore birds had access to cached food in addition to the feeding platforms.

The increased length in both the Hp and NCL along with increased branching in the NCL one week after hearing predator vocalizations suggests that birds are retaining an enduring memory of fear. Interventions to enhance spatial memory increased dendritic length in the avian Hp (Freire and Cheng 2004) and in both the hippocampus and prefrontal cortex in rodents (Mu et al. 2022). Increased dendritic length and

branching in the Hp has also been demonstrated in female brown-headed cowbirds under semi-natural conditions in the same aviaries during the spring breeding season (Witterick 2017). The avian NCL is an important region for learning-induced plasticity in mature neurons, with learning corresponding to increased dendritic complexity in corvids (Parishar et al. 2024) and is well known for its role in working memory and decision making (Li et al. 2020). In contrast, decreased neuron complexity in both the Hp and caudal nidopallium has also been shown in conjunction with decreased learning performance in corvids (Taufique et al. 2018). Enhanced learning and spatial memory in response to predator exposure may be particularly important for wild animals proactively responding to varying levels of predation risk across the landscape of fear (Laundré et al. 2010, Creel 2018) to avoid areas with a higher likelihood of future predator encounters. In rodent models, increased activity in the prefrontal cortex, analogue to the avian NCL (Herold et al. 2011), has been shown to correspond to increased fear discrimination (Day et al. 2020). If increased dendritic length and branching also increased neuronal activity, this may allow animals to better discriminate different degrees of predation risk, minimize false alarms, and reduce unnecessary energy expenditure in the harsh winter environment.

The contrast between my lack of enduring effects on Δ FosB and neurogenesis under semi-natural conditions compared to previous laboratory studies in the same species (Zanette et al. 2019, Chapter 2) implies that captivity and the environment in which an animal experiences predator-induced fear may also play an important role in the neural response. In the lab, we previously found increased Δ FosB activation in the hippocampus (Hp) and medial ventral arcopallium (AMV) of chickadees (Zanette et al. 2019). In contrast, the lack of enduring effect on Δ FosB presented here is consistent with my previous findings manipulating predation risk for brown-headed cowbirds in the spring breeding season in the same aviaries (Witterick 2017). The differences in Δ FosB activation in response to predator vocalizations in the lab compared to in the large outdoor aviaries suggests that captivity and the laboratory environment, with its lack of refuge or ability to otherwise escape from predators, may play a role in Δ FosB activation in response to predator induced fear. When looking at neurogenesis, I found no enduring

effects in the chickadees for either cell proliferation (PCNA) or immature neurons (DCX). I previously demonstrated enduring suppression of DCX in the chickadee AMV and PCNA in the Hp of male chickadees (Chapter 2) in the lab, along with suppressed DCX in the Hp and AMV of brown-headed cowbirds who heard predator vocalizations under semi-natural conditions in the spring breeding season (Witterick 2017). Allowing chickadees to perform natural behaviours such as food caching may have also counteracted any expected predator induced suppression of neurogenesis (Tanapat et al. 2001, Dunlap et al. 2017, Vignisse et al. 2017, Witterick 2017), as chickadees have previously demonstrated increased hippocampal neurogenesis as new memories are formed in relation to food cache locations (LaDage et al. 2010). Consistent with my lack of enduring effect of fear on Δ FosB and neurogenesis, mountain chickadees show behavioural alterations indicative of reduced cognitive flexibility under harsh environmental conditions (Croston et al. 2017). The contrast between the laboratory and semi-natural manipulations suggests that minimizing the influence of captivity is needed to extrapolate the enduring effects of fear measured in wildlife in the laboratory environment to their free living counterparts (Clinchy et al. 2011, Cohen et al. 2023).

Overall, I found that chickadees reduced foraging when exposed to predator calls, with enduring alterations to dendritic morphology in the Hp and NCL indicative of an enduring memory of fear. Alterations to dendritic morphology in response to fear conditioning have been found to persist for at least 5 weeks without interventions promoting fear extinction (Heinrichs et al. 2013), suggesting that fear-induced alterations may be more persistent for dendritic morphology than the other markers measured. Even when dendritic morphology does recover after chronic stress, the dendritic architecture often remains altered as new growth occurs on different dendrites than those that atrophied, and the likelihood of recovering from stress induced alterations to dendritic morphology decreases with age (McEwen and Morrison 2013). In contrast, the half-life of PCNA is 20 h in rodents (Bravo and Macdonald-Bravo 1987) and the half-life of Δ FosB is 8 days (Robison and Nestler 2022), making these effects more dynamic in response to predator-induced fear but also more susceptible to additional variation from a constantly changing environment like that experienced in the semi-

natural aviaries. Conventional laboratory animal housing has been associated with increased anxiety like behaviour and reduced survival when compared to enriched housing, both of which are also seen in response to predator induced fear and raise concerns about potential confounding effects of captivity on the neural response to fear (Clinchy et al. 2011). While the aviaries provide an enriched environment compared to the laboratory, chickadees lacked the ability to move to a presumably lower risk environment. To determine whether comparable enduring alterations to dendritic morphology in response to predator-induced fear can be found outside of the confines of captivity, the logical and necessary next step is experimental manipulation of perceived predation risk in free-living animals.

Table 3.1: List of species used in the auditory playbacks for the chickadees. Predator and non-predator species were matched based on their acoustic call characteristics (frequency and maximum amplitude).

Time	Predators	Non-Predators
Day	Cooper's hawk, <i>(Accipiter cooperii)</i>	Song sparrow <i>(Melospiza melodia)</i>
Day	American crow <i>(Corvus brachyrhynchos)</i>	Mallard <i>(Anas platyrhynchos)</i>
Day	Red-tailed hawk <i>(Buteo jamaicensis)</i>	Blue jay <i>(Cyanocitta cristata)</i>
Day	Merlin <i>(Falco columbarius)</i>	Downy woodpecker <i>(Picoides pubescens)</i>
Day	Sharp-shinned hawk <i>(Accipiter striatus)</i>	Hairy woodpecker <i>(Picoides villosus)</i>
Night	Northern saw-whet owl <i>(Aegolius acadicus)</i>	Wood frog <i>(Lithobates sylvaticus)</i>
Night	Barred owl <i>(Strix varia)</i>	Northern leopard frog <i>(Lithobates pipiens)</i>

Table 3.2: Ethogram of all behaviours measured in the recorded videos

Behaviour	Definition
Moving	Moving around the cage (flying, walking, hopping)
Vigilance	Head up, immobile (may still be moving head looking around)
Feeding	Head down, consuming or handling food from the lard block
Chased	Supplanted or chased opponent
Resist	Resist supplanting by opponent
Submissive	Elicited submissive posture in opponent
Wait	Fed while opponent waited or arrived on platform immediately after previous individual left (<1s)

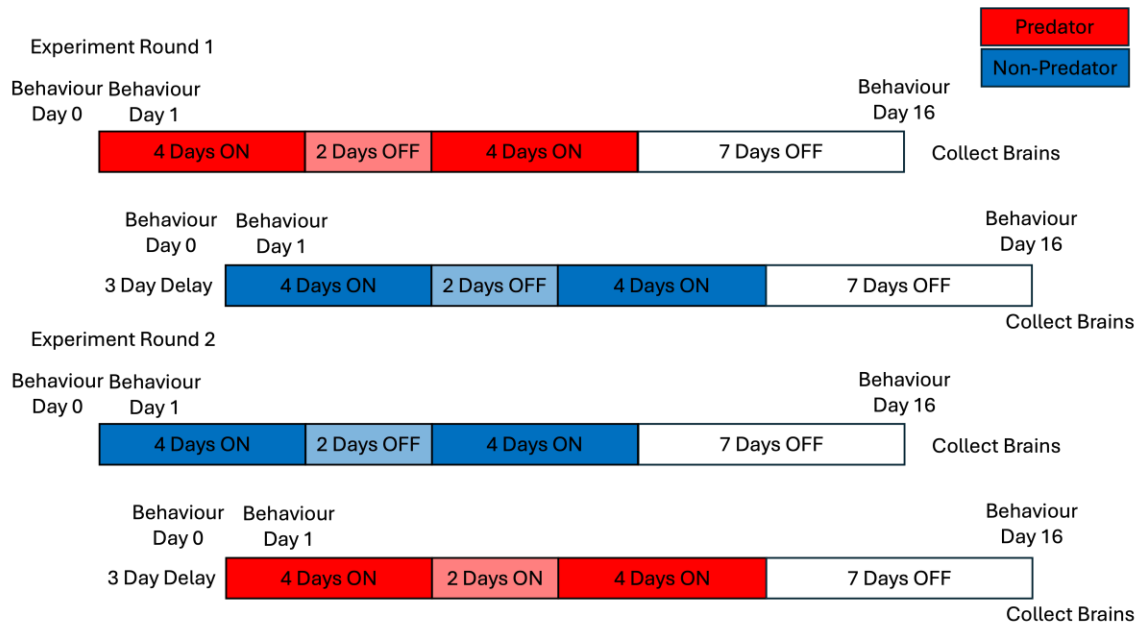


Figure 3.1: Graphic overview of the experimental design including the playback schedule, behavioural observation timepoints, and the brain collection.

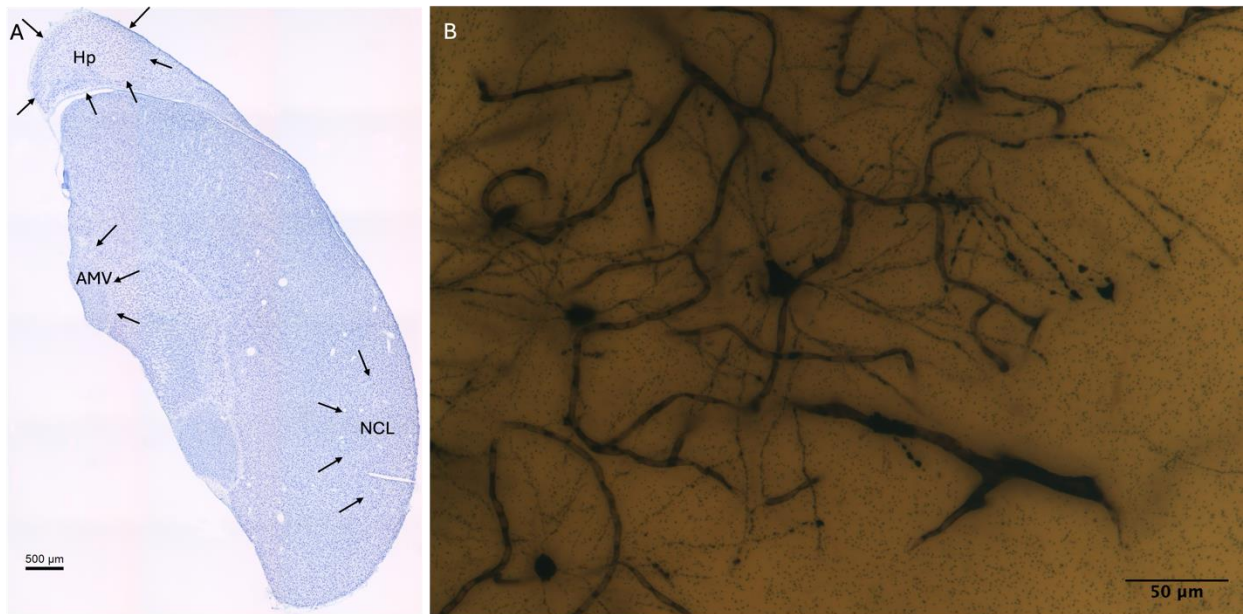


Figure 3.2: A) Photomicrograph of a Nissl stained coronal section of chickadee telencephalon. Arrows depict boundaries of areas where neurons were photographed. B) Example of the Golgi-Cox staining in the chickadee hippocampus.

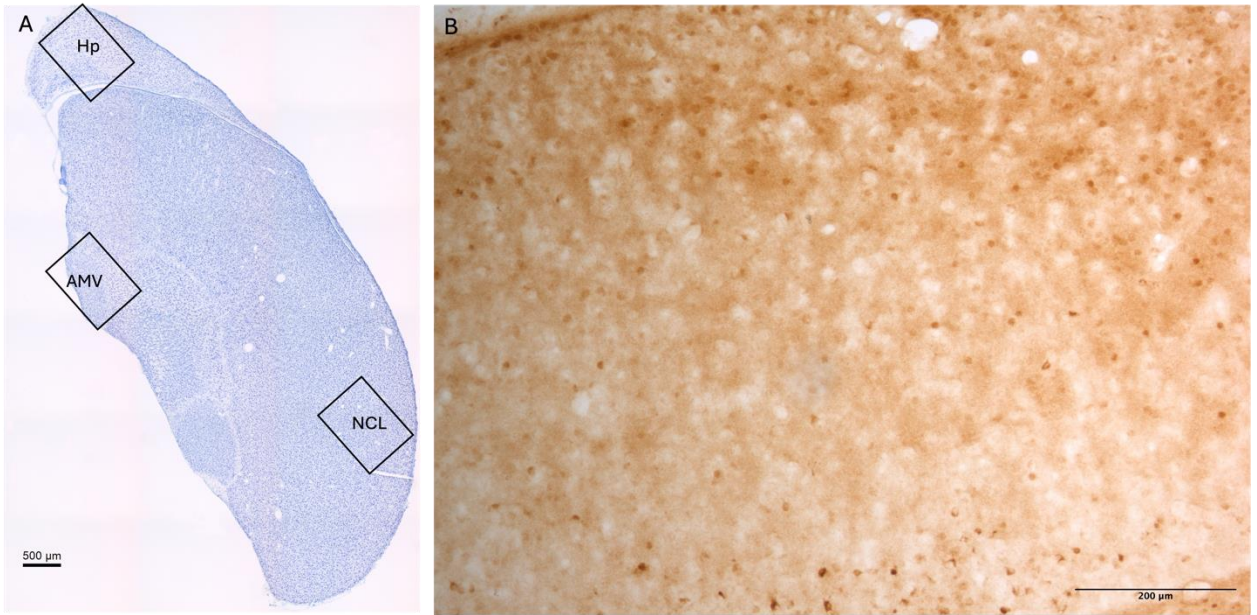


Figure 3.3: A) Photomicrograph of a Nissl stained coronal section of chickadee telencephalon. Boxes depict locations to scale where images were taken for Δ FosB quantification. B) Higher magnification photomicrograph of Δ FosB immunoreactivity taken in the Hp.

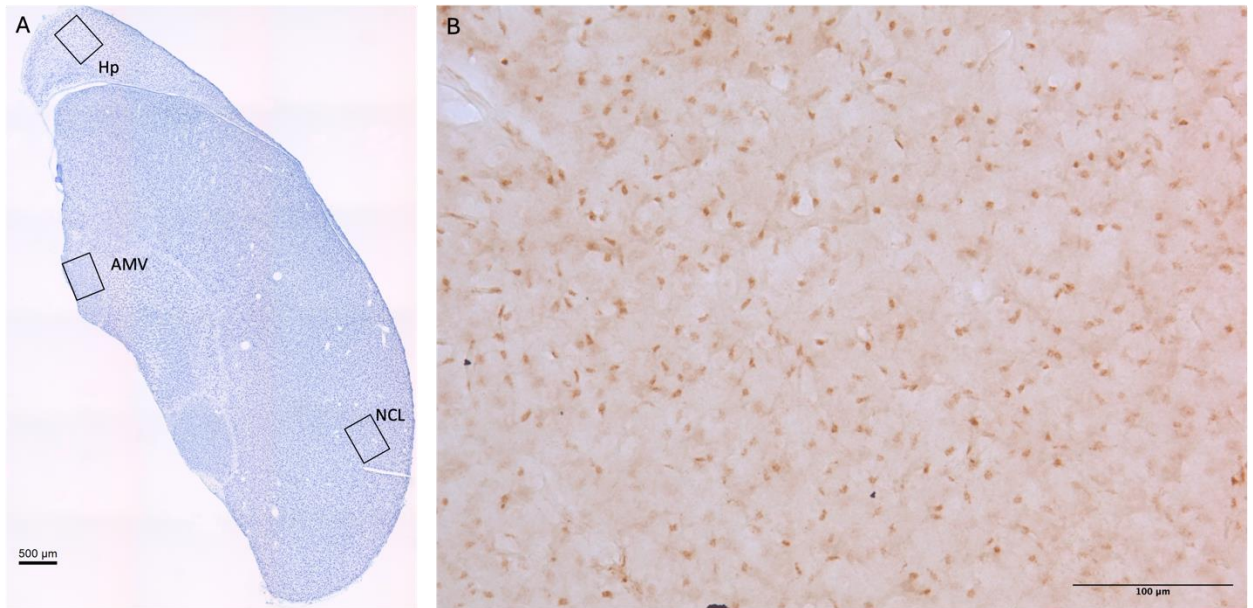


Figure 3.4: A) Photomicrograph of a Nissl stained coronal section of chickadee telencephalon. Boxes depict locations to scale where images were taken for PCNA quantification. B) Higher magnification photomicrograph of PCNA immunoreactivity taken in the Hp.

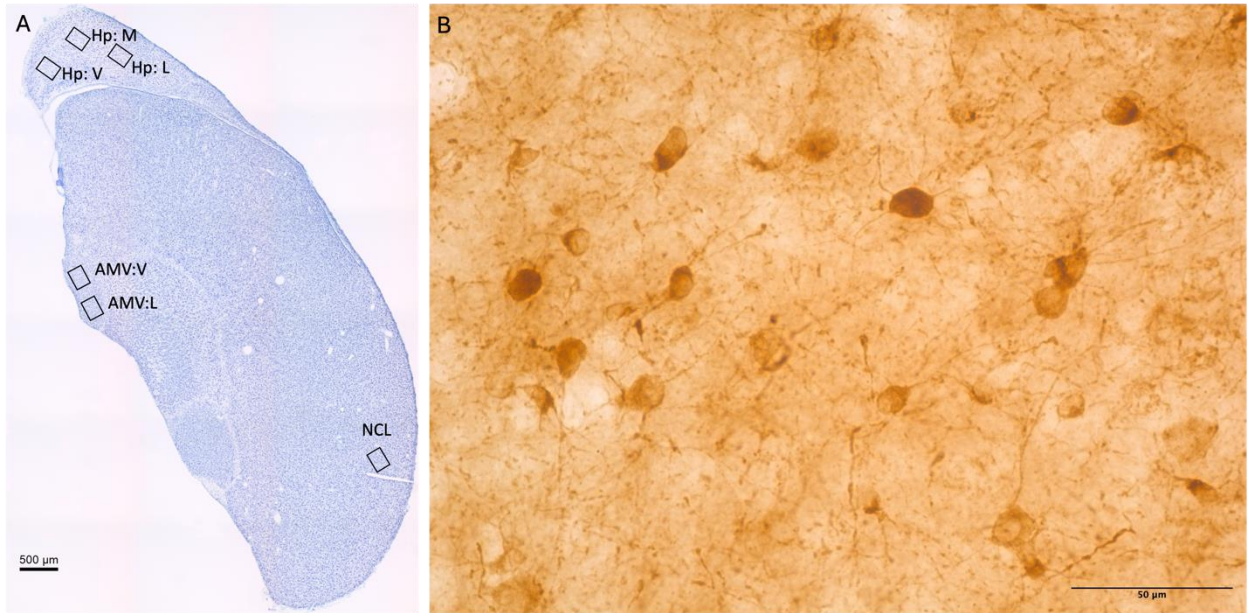


Figure 3.5: A) Photomicrograph of a Nissl stained coronal section of chickadee telencephalon. Boxes depict locations to scale where images were taken for DCX quantification within each brain region measured (L: lateral, M: medial, V: ventral). B) Higher magnification photomicrograph of DCX immunoreactivity in the Hp.

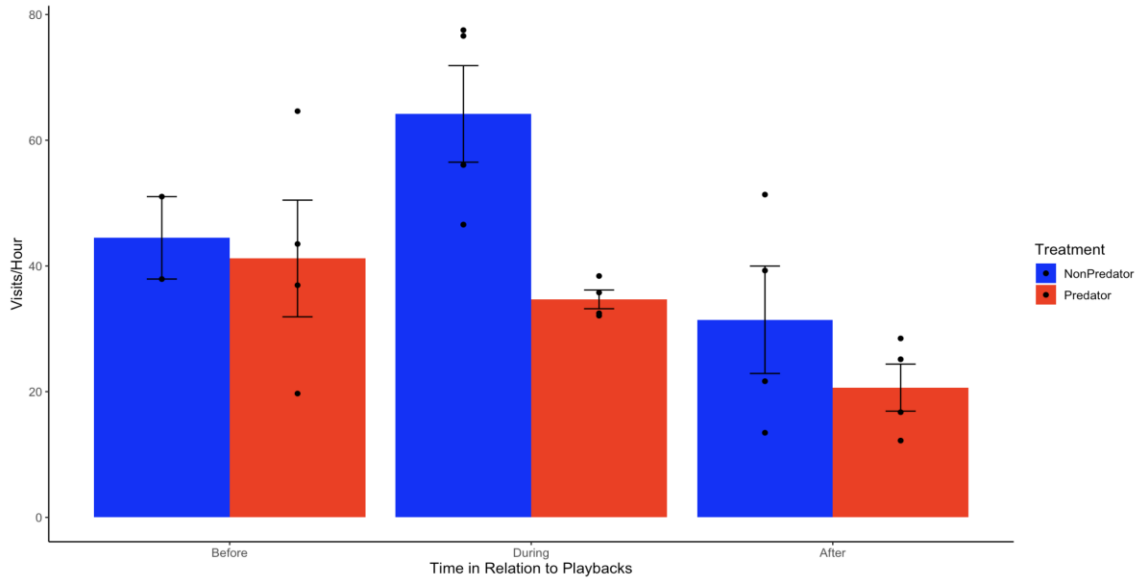


Figure 3.6: The number of visits per hour made to a feeding platform before ($p=0.25$), during ($p=0.048$), and after ($p=0.43$) playbacks. $N=8$ Aviaries (NP=4; Pred=4). Bars indicate means \pm standard error, filled circles indicate individual data.

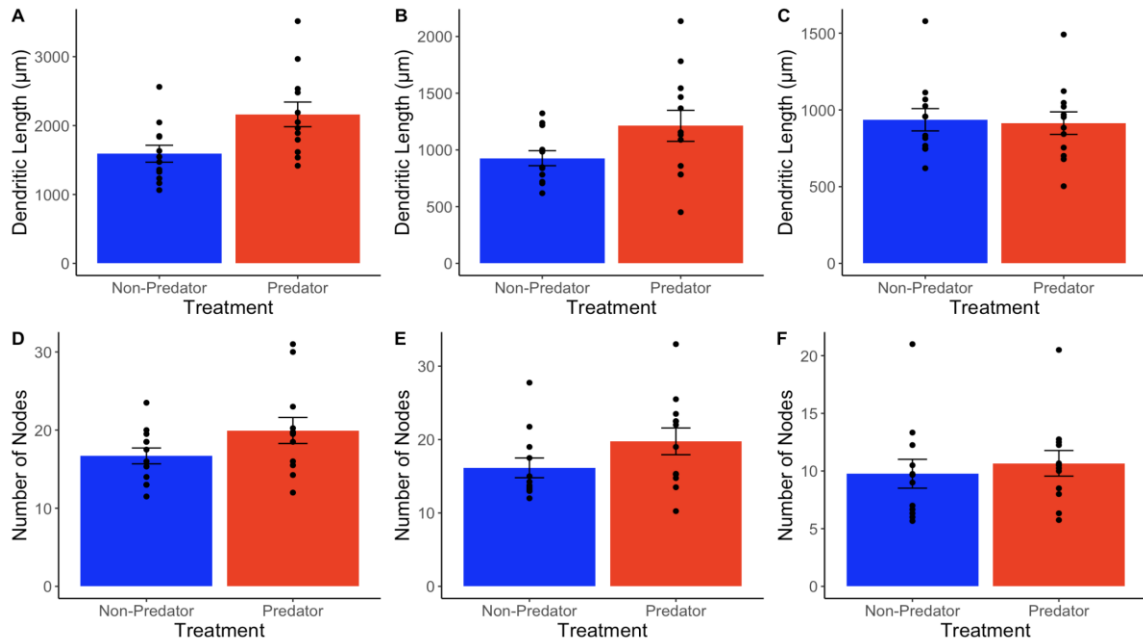


Figure 3.7: The effect of playback treatment on the total dendrite length of neurons in A) hippocampus ($p=0.047$), B) caudolateral nidopallium ($p=0.0087$), and C) medial ventral arcopallium and the number of branch nodes in the D) hippocampus, E) caudolateral nidopallium ($p=0.03$), and F) medial ventral arcopallium. $N=24$ (NP: 5F, 7M; Pred: 3F, 9M). Bars indicate means \pm standard error, filled circles indicate individual data.

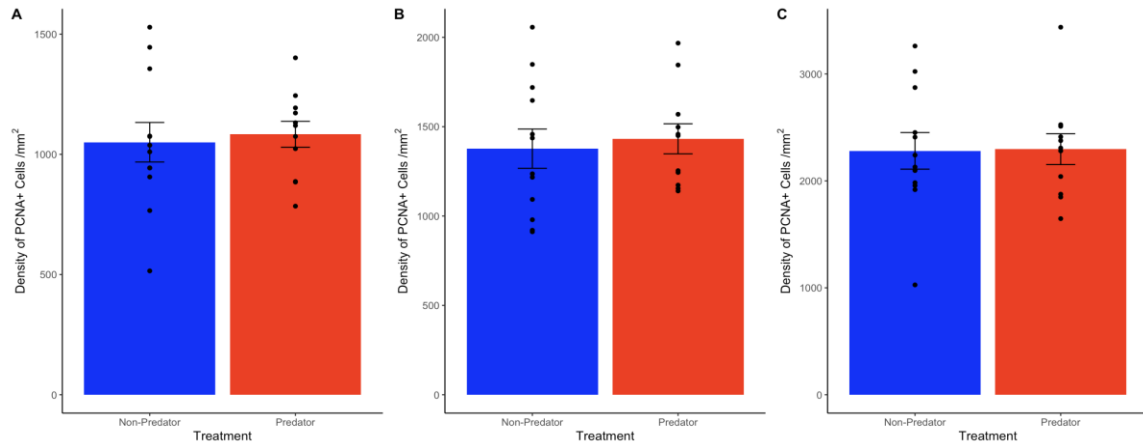


Figure 3.8: The effect of playback treatment on the density of PCNA-ir cells in the A) hippocampus, B) caudolateral nidopallium, and C) medial ventral arcopallium. N=23 (NP: 5F, 7M; Pred: 4F, 7M). Bars indicate means \pm standard error, filled circles indicate individual data.

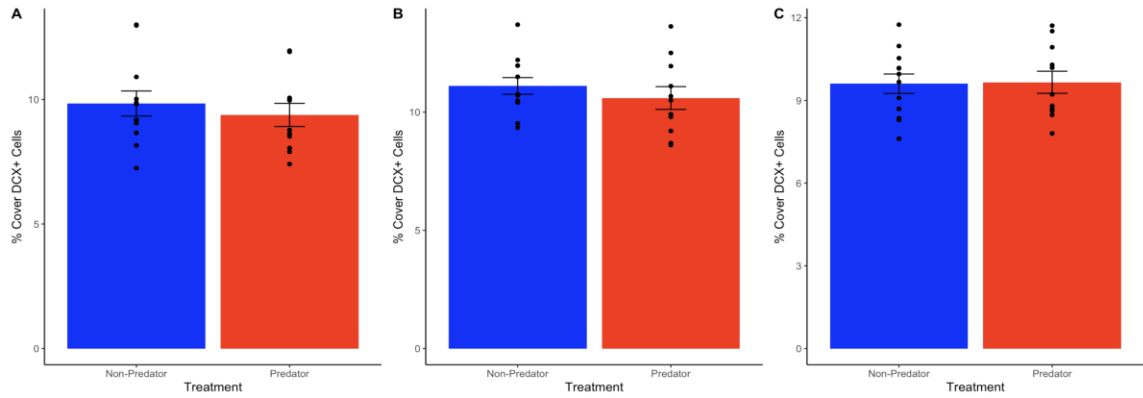


Figure 3.9: The effect of playback treatment on the percent cover of DCX-ir cells in the A) hippocampus, B) caudolateral nidopallium, and C) medial ventral arcopallium. N=23 (NP: 5F, 7M; Pred: 4F, 7M). Bars indicate means \pm standard error, filled circles indicate individual data.

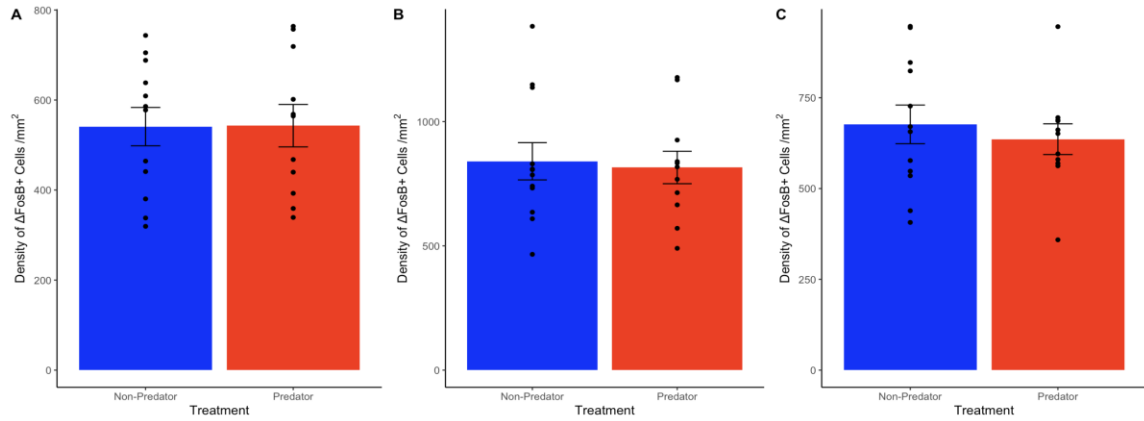


Figure 3.10: The effect of playback treatment on the density of Δ FosB-ir cells in the A) hippocampus, B) caudolateral nidopallium, and C) medial ventral arcopallium. N=23 (NP: 5F, 7M; Pred: 4F, 7M). Bars indicate means \pm standard error, filled circles indicate individual data.

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Chapter 4

4 Fear of predators has enduring effects on the brain in free-living rodents

4.1 Introduction

Predator encounters are a ubiquitous experience among wildlife, where animals must balance the need to eat and find mates with the need to avoid being eaten and survive another day. Predator-induced fear has been demonstrated to alter wildlife behaviour, leading to multi-generational demographic consequences from reduced fecundity and offspring survival (Zanette et al. 2011, Allen et al. 2022). In conjunction with these established behavioural and demographic effects of fear observed in the wild, enduring effects on neurobiology, indicative of an enduring memory of fear, have been demonstrated in wildlife in the laboratory (Zanette et al. 2019, Chapter 2) and living in semi-natural conditions in outdoor aviaries (Witterick 2017, Chapter 3). While captive studies tell us a great deal about the enduring effects of fear on neurobiology, there is always a possibility that the observed effects may be influenced in some part by the unnatural conditions of captivity such as lack of sufficient refuge or the inability to leave for an area that is perceived to be lower risk (Clinchy et al. 2011). While I would expect to see comparable enduring effects of fear on the brain in free-living wildlife, experimental manipulations are a necessary next step to understand how fear affects the brain without any potential interaction that could be ascribed to a by-product of captivity rather than the fear of predators.

The tools for quantifying the enduring effects of fear on neurobiology in free-living wildlife arose from the convergence of interests between ecologists studying predator-prey interactions and biomedical research on post-traumatic stress disorder (PTSD; Clinchy et al. 2011). Predators or their cues (such as mounts, scent, or sounds) are commonly used tools to induce PTSD-like symptoms in laboratory models for PTSD due to the robust behavioural and biological phenotypes produced and the ecological validity from a cue that is life-threatening but produces no physical harm (Deslauriers et al. 2018). In humans, a PTSD diagnosis requires enduring, disruptive symptoms

associated with the memory of experiencing a traumatic event (American Psychiatric Association 2013), while laboratory animal models define the criteria for an enduring, PTSD-like effect as effects persisting for at least one week in a small rodent (Deslauriers et al. 2018). Although these models provide valuable tools for the quantification of enduring effects of fear on neurobiology, the traditional focus on captive models using only male rodents raises additional questions as to how these effects would present in the female half of the animal population (Deslauriers et al. 2018). Given that sex differences are commonly observed in laboratory rodent behavioural models and suggested to represent sex differences in adaptive strategies for survival (Shansky 2018), quantifying the enduring effects of fear in both sexes is crucial to understanding the enduring effects of fear on behaviour and neurobiology in free-living wildlife.

Laboratory animal models for PTSD and previous predation risk manipulations in wild caught animals in both laboratory and semi-natural conditions (Witterick 2017, Zanette et al. 2019, Chapters 2, 3) provide the foundations to quantify the enduring effects of fear on neurobiology through changes to activation, neurogenesis, apoptosis, and neuron structure. Enduring effects on activation are defined for this thesis as an enduring marker of earlier immediate early gene expression and can be measured using Δ FosB, a uniquely stable member of the FOS family of immediate early gene transcription factors with a half-life of up to 8 days (Robison and Nestler 2022). Induction of Δ FosB in the basolateral amygdala and infralimbic area of the prefrontal cortex has been demonstrated to promote resilience against social defeat stress, while Δ FosB induction in the hippocampus is critical for learning, memory, and neurogenesis (Vialou et al. 2014, Manning et al. 2019, Robison and Nestler 2022). Enduring increases in Δ FosB in response to predator cues have been reported under laboratory conditions in both rodents and wild caught songbirds (Staples et al. 2009, Mackenzie et al. 2010, Zanette et al. 2019). Neurogenesis can be quantified in immature neurons using doublecortin, a microtubule-associated protein expressed in migrating neuronal precursor cells (Couillard-Despres et al. 2005). Experimentally elevated neurogenesis after a traumatic event has been shown to weaken trauma memories and reduce PTSD-like behaviours, suggesting that suppressed neurogenesis may play an important role in the persistence of fear memories (Fujikawa et al. 2024). Apoptosis can be quantified with

activated caspase-3, a key mediator of both the intrinsic and extrinsic pathways of apoptosis involved in neurodevelopment, synaptic plasticity, and alterations to neuron morphology (Chen et al. 2020, Asadi et al. 2022). Enduring increases in apoptosis have been reported in the prefrontal cortex, amygdala, and hippocampus using the single prolonged stress model for PTSD (Xiao et al. 2011, Yu et al. 2014, Han et al. 2021). Fear induced changes to neuron structure can be measured through changes to dendritic morphology, often through changes to dendritic length and branching. Alterations to dendritic morphology in lab rodents have been demonstrated in the hippocampus (Hoffman et al. 2016, Cohen et al. 2020, 2023) and basolateral amygdala (Mitra et al. 2009, Adamec et al. 2012) in response to predators or their cues while comparable effects have been demonstrated in response to restraint stress in the prefrontal cortex (Liston et al. 2006, Radley et al. 2006, Garrett and Wellman 2009).

In this experiment I exposed free-living meadow voles (*Microtus pennsylvanicus*; hereafter 'voles') to predator calls in their natural habitat, allowing animals to engage in their natural anti-predator behaviours and removing any potential interaction between captivity and the fear response. Building on previous laboratory and semi-natural manipulations, I quantified the enduring effects of the fear of predators on activation, neurogenesis, apoptosis, and dendritic morphology in three brain regions (the prefrontal cortex, amygdala, and hippocampus) that are commonly associated with fear processing (outlined in Chapter 1, Figure 1.1). To corroborate the enduring effects of fear in the brain, I also performed a behavioural assay comparing the time of entry in live traps, which I use as a measure of nocturnal activity. Rodents have been demonstrated to be more vigilant, forage less, and spend less time overall in foraging patches that are perceived to be at a higher risk of predation (Farnworth et al. 2016, Zhang et al. 2020, Hoffmann et al. 2022, Ferreira et al. 2023), all indicators that nocturnal activity is reduced when predation risk is increased. My results demonstrate that the fear of predators has enduring effects on behaviour, neuron structure, neurogenesis, activation, and apoptosis, indicative of an enduring memory of fear and that sex plays an important role in the enduring response to fear.

4.2 Methods

4.2.1 Study Species

I used meadow voles to study the enduring effects of fear of predators on the brain in free living wildlife. Voles were selected for this chapter for their abundance in Southern Ontario, their relatively small home range, their trapability, and their use as a model organism for neurobiology studies.

4.2.2 Study Sites

This experiment was conducted at two field sites in Southern Ontario, the Environmental Sciences Western (ESW) Field Station near London, Ontario and rare Charitable Research Reserve (RARE) in Cambridge, Ontario. Each site contained two experimental grids at least 200 m apart and separated by a natural barrier, that being an agricultural drainage channel at ESW and Cruickston Creek at RARE (Figure 4.1A). Grids were set up on meadow areas of each property, each containing 49 Longworth live traps arranged in a 7 x 7 configuration (Figure 4.1B). Grids were 45 m by 45 m with trap locations every 7.5 m marked by flag stakes and flagging tape on taller vegetation. Traps were setup and locked open approximately three weeks before trapping began to allow voles to explore and scent mark the traps.

4.2.3 Mark Recapture Trapping

Prior to the start of experimental playbacks, two (RARE) or three (ESW) days of mark-recapture were conducted to determine the population size of each grid and the residency location of animals being trapped, to ensure that voles were not dispersing between the experimental grids. For the mark-recapture trapping, traps were lined with cotton bedding for warmth and baited with oats and apple slices. Traps were set between 16:00-19:00 and checked beginning at 8:00 the following morning. All animals caught were tagged on each ear (Stoelting Co ear tags for mice), weighed, and sex determined. Animals were then released at the point of capture. 119 voles were tagged at the ESW and 31 voles were tagged at RARE. The traps were locked open during the day between

trapping periods and throughout the experimental manipulation to remove the novelty of the trap presence.

4.2.4 Predation Risk Manipulation

The experiment was conducted between 26 September and 6 November 2022. To manipulate perceived predation risk, audio playbacks of either predator or non-predator species were broadcast over 11 days on a 4 days on, 2 days off, 5 days on cycle, which was then followed by 7 days of no audio to meet the criteria for enduring effects. Each grid contained two battery operated speaker units, each containing a set of speakers and MP3 players enclosed in a weatherproof wooden box, and mounted to posts approximately 1.5 m tall at the outer corners of the grid (Figure 4.1C). Speakers were located at the NW and SE corners of the grid for the first four days of playbacks and then switched to the NE and SW corners for the remaining five days. Playbacks consisted of 6 species of either predator or non-predator species (Table 4.1) broadcast at the ecologically relevant time of day. Species were matched between treatments for their audio characteristics, following Zanette et al (Zanette et al. 2011). Playbacks were broadcast at 80 dB SPL from 1 m away using a call to silence ratio of 1:1.5 during the day and 1:2.3 at night. All calls were obtained from the Macaulay Library Database (Cornell University Lab of Ornithology, Ithaca, New York, USA) and the Xeno-Canto foundation (www.xeno-canto.org).

4.2.5 Brain Processing

Seven days after the end of playbacks, brains were collected for the neurobiology assays. Traps were set between 16:00 – 17:00 the evening before, and were lined with cotton bedding, and baited with oats and apple slices. Traps were also equipped with two iButton temperature loggers enclosed in mesh window screening, one inside the trap and one outside, to determine the time of entry in the trap (Orrock and Connolly 2016). Upon capture, ear tags were recorded if present, animals were weighed, and sex was determined. Only adult voles were euthanized, which was determined as voles with a mass >25 g (McShea and Madison 1986, Dobson and Myers 1989). Juveniles and any females that were pregnant or nursing (nipples protruding) were released at the point of

capture. Voles were euthanized on site by overdose of isoflurane and assigned alternately to be processed for immunohistochemistry or dendritic morphology while maintaining a balanced sex ratio for each grouping. Immediately following euthanasia and decapitation, blood samples were collected from the carotid artery using heparinized micro capillary tubes. Brains for immunohistochemistry were then removed and fixed in 4% paraformaldehyde for a minimum of 7 days, then cryoprotected in 30% sucrose solution for 48 h until saturated and frozen at -80 °C for long term storage. Brains for dendritic morphology were removed and incubated in Golgi-Cox Solution (1% potassium dichromate, 0.8% potassium chromate, and 1% mercuric chloride in water) in the dark for 25 days, then transferred to 30% sucrose for 48 h until saturated and frozen at -80 °C for long term storage. Blood samples were spun on a centrifuge to separate the plasma and red blood cells, which were frozen for long term storage. A total of 46 brains were collected, with 24 processed for immunohistochemistry and 22 for dendritic morphology.

4.2.6 Golgi Staining and Neuron Tracing

Golgi-Cox stained brains (22 voles; NP: 6F, 7M; Pred: 5F, 4M) were sliced to 500 µm coronal slices using a vibratome. I started collecting slices at the end of the start of the prelimbic area, and stopped after the corpus collosum was fully connected between hemispheres. I started collecting slices again when the hippocampal formation became visible and stopped when the brainstem extended lower than the lobes of the cerebrum. Brains were processed following Louth et al. (Louth et al. 2017). Brains were incubated overnight in 6% sucrose solution at 4 °C, then developed in 2% paraformaldehyde, followed by 2.7% ammonium hydroxide, and then 10% Kodak Fixative A, with slices washed in distilled water between each step. All imaging was done in NeuroLucida, with image stacks taken using a 30X objective lens for 4 pyramidal neurons in each of the basolateral amygdala, layer 2/3 of the cingulate cortex, and the CA1 sublayer of the hippocampus (Figure 4.2). All 22 individuals had neurons photographed from the basolateral amygdala and cingulate cortex, while a subset of 12 individuals had CA1 neurons photographed due to variation in the Golgi-Cox staining in the hippocampus. Neurons were selected from the two slices where the region of interest was present, with one neuron selected from each hemisphere of that slice. To be selected, neurons had to be

fully visible within the slice of interest and not obscured by any other neuron or blood vessel. Each image stack took a photo at 1 μ m intervals to photograph the entire neuron, ranging from 50-150 photos depending on the size of the individual neuron. If the neuron exceeded the frame of view in the x or y plane, additional photo stacks were taken as needed and stitched together in ImageJ (Schneider et al. 2012) prior to tracing. Neurons were traced using Neuromantic (Myatt et al. 2012), with reconstructions converted back to Neurolucida files using Neuronland. Data was then exported from Neurolucida and converted from pixels to μ m for analysis.

4.2.7 Immunohistochemistry and image analysis

For immunohistochemistry, brains (24 voles; NP: 7F, 7M; Pred: 5F, 5M) were sectioned to 40 μ m coronal slices using a cryostat, taking four series starting from the prelimbic area to where the corpus collosum was fully connected between hemispheres, then collecting again when the hippocampal formation became visible and stopping when the brainstem extended lower than the lobes of the cerebrum. I carried out immunohistochemistry on free floating sections to label Δ FosB (FosB (102) rabbit IgG, sc-48, Santa Cruz Biotechnology), doublecortin (DCX (C-18) goat IgG, sc-8066, Santa Cruz Biotechnology), and activated caspase 3 (Caspase-3, rabbit IgG, #13847, Abcam) following established protocols (Zanette et al. 2019, Diez et al. 2021), with the primary anti-body at a concentration of 1:500, 1:250, and 1:500 in 0.3% phosphate-buffered saline with triton (PBS/T), respectively. Sections were then labelled with a secondary antibody (goat anti-rabbit at 1:250 for Δ FosB and activated caspase-3 and horse anti-goat at 1:400 for DCX, Vector Laboratories) and visualized with diaminobenzidine solution.

For each marker, I quantified immunoreactivity in the hippocampus, amygdala, and prefrontal cortex (Figure 4.3). For the hippocampus, z-stack images were taken of the granular layer of the dentate gyrus and the CA1 and CA3 layers of the cornu ammonis. For the amygdala, z-stack images were taken of the basolateral amygdala and central amygdala. For the prefrontal cortex, z-stack images were taken of the prelimbic area, the infralimbic area, and the anterior cingulate cortex. The z-stack took images covering the depth of the slice of interest, then the photos were combined in the Leica

Application Suite software to produce a single image with the entire field in sharp focus regardless of the depth within the slice. All images were taken starting from the anterior end of the region, taking one photo for each subregion of interest for the first five slices with both hemispheres present on the slide. For Δ FosB and activated caspase-3, all images were taken using a 20X objective lens. ImageJ (Schneider et al. 2012) was calibrated to the image measurement to measure the area of interest in mm^2 . I converted the images from colour to 16-bit greyscale, subtracted the background, and enhanced the contrast. I then used the thresholding tool to convert the Δ FosB or activated caspase-3 immunoreactive nuclei to black cells on a white background and used the count function to quantify the number of cells. Density was calculated as the Δ FosB or activated caspase-3 immunoreactive cells/ mm^2 for each photograph. For DCX, all images were taken using a 40X objective lens. I converted the images from colour to 16-bit greyscale, enhanced the contrast, then used the thresholding tool in ImageJ (Schneider et al. 2012) to convert the DCX positive cells and fibres to black against a white background and quantified the percent cover of DCX immunoreactive cells. All images were collected and quantified without knowledge of the treatment groups or sex to avoid bias in the results.

4.2.8 Quantifying Trap Entry and Handling Time

Two iButton temperature loggers, wrapped in a strip of aluminum window screen to hold one inside and one outside of each Longworth trap, were used to calculate the time animals entered the trap (Orrock and Connolly 2016). The iButtons were set to log the temperature every 10 min. A breakpoint analysis was calculated to determine the time at which the temperature points deviated between the two iButtons to determine when the animal entered the trap. In cases where there was no deviation between the iButtons or an iButtons was removed in the field, a control was selected for comparison for each trapping evening from a trap where no animal was captured. The time of entry from the breakpoint analysis was then used in conjunction with the recorded time of trap opening and euthanasia from the field to calculate the time each animal spent in trap along with the total handling time from trap entry to euthanasia for analysis. There was no

difference between treatments in the total time spent in the traps for voles used in the neurobiology assays (Mann-Whitney U, $W=293.5$, $p=0.189$).

4.2.9 Processing Blood Samples for Corticosterone

Plasma corticosterone concentration was measured using a commercial enzyme linked immunosorbent assay kit according to kit instructions (Corticosterone ELISA kit, ADI-900-097, Enzo Life Sciences), which has previously been validated for use in the closely related prairie vole (*Microtus ochrogaster*; McNeal et al. 2017).

4.2.10 Statistical Analysis

When analyzing treatment effects on the time of entry into the traps, all captured animals were included in the analysis including those that were later released ($N=69$ voles; $NP=44$; $Pred=25$). A Cox proportional hazards model was used to analyze the effect of treatment on trap entry time, measured from 17:00 when the traps were opened to the endpoint of entering the trap. For the immunohistochemistry assays, the average for each marker was calculated for each brain subregion, to give one value per brain subregion per individual for each of Δ FosB, DCX, and activated caspase-3. These were analyzed for each brain region using generalized estimating equations, with treatment and sex as fixed factors, and subregion as the within subject effect to account for the repeated measures within the region. Post hoc analyses were conducted using Fishers least significant difference with an alpha value of 0.0125 to control for multiple tests. Capture time, as measured from the time voles entered the trap until the time of euthanasia, was included as a covariate in all models to begin and removed when it was not a significant covariate to increase the model power. For neuron structure, the average for each individual was calculated for length and branching for the apical and basal dendrites. Neuron length and branching were each analyzed using a repeated measures ANOVA, with treatment and sex as the fixed factors and dendrite type (apical or basal) as the repeated factor. Corticosterone was analyzed using quantile regression with $\tau=0.5$ for a median regression to accommodate for data that did not meet the assumptions for ANCOVA. The mean corticosterone concentration (pg/mL) from two replicate wells was analyzed with treatment and sex as fixed factors and capture time as the covariate.

4.3 Results

4.3.1 Behavioural assay and physiology

Voles who heard predator playbacks one week earlier entered the live traps later in the night compared to their non-predator counterparts, determined by a Cox proportional hazard model (Figure 4.4; Wald=4.63, $p=0.0313$). The time of trap entry was not significantly correlated to temperature at entry (Spearman rank correlation $p>0.5$). While behaviour indicated an enduring effect of fear, we found no enduring effect on physiology as measured by plasma corticosterone (Predator: 21556.09 ± 5408.718 ; Non-predator: 15734.57 ± 2346.280 ; mean pg/mL corticosterone concentration \pm SE; main effects of treatment, sex, and interaction all $p>0.6$; time in trap was not a significant covariate $p=0.57$).

4.3.2 Neuron Structure

Hearing predator calls one week earlier had enduring effects on neuron structure in the amygdala. Voles who heard predators one week earlier had longer (Figure 4.5B; $F_{1,18}=7.28$, $p=0.015$) and more branched (Figure 4.5E; $F_{1,18}=7.063$, $p=0.016$) dendrites in the amygdala, with no effect of or interaction with sex (all $p>0.1$). There was no enduring effect of treatment, sex or interaction for dendritic length or branching in either the PFC (Figure 4.5A, 4.5D; all $p>0.2$) or the Hp (Figure 4.5C, 4.5F; all $p>0.4$).

4.3.3 Neurogenesis

Fear of predators had an enduring effect on neurogenesis, as measured by DCX immunoreactivity, with a treatment x sex interaction (Figure 4.6A; Wald $X^2=15.52$, $p<0.001$) along with a main effect of treatment (Wald $X^2=6.21$, $p=0.013$) in the prefrontal cortex. This effect was driven by the male voles, where males who heard predators one week earlier had significantly greater DCX immunoreactivity than males who heard the non-predator controls (LSD $p<0.001$) and females in the predator treatment (LSD $p<0.001$). There was no main effect or interaction of treatment or sex in the amygdala (Figure 4.6B, all $p>0.3$) or hippocampus (Figure 4.6C, all $p>0.6$) along with no main effect of sex in the prefrontal cortex ($p=0.2$).

4.3.4 Neural activation

Fear of predators had an enduring effect on activation, as measured by Δ FosB immunoreactivity, with a treatment x sex interaction (Figure 4.7A; Wald $X^2=4.57$, $p=0.032$) in the vole prefrontal cortex, whereby males in the predator treatment have greater Δ FosB immunoreactivity than their female counterparts (Fisher LSD; $p=0.012$). There was no corresponding main effect of treatment or sex (all $p>0.1$). There was no main effect or interaction of treatment or sex in the amygdala (Figure 4.7B; all $p>0.2$) or the hippocampus (Figure 4.7C; all $p>0.5$) for Δ FosB immunoreactivity.

4.3.5 Apoptosis

Apoptosis, as measured by activated caspase-3 immunoreactivity, was significantly altered by fear. There was a significant treatment x sex interaction in the prefrontal cortex (Figure 4.8A; Wald $X^2=11.51$, $p<0.001$), amygdala (Figure 4.8B; Wald $X^2= 11.12$, $p<0.001$), and hippocampus (Figure 4.8C, Wald $X^2 = 10.39$, $p=0.001$), with capture time as a significant covariate for all three regions (PFC: Wald $X^2=16.80$, $p<0.001$; Amg: Wald $X^2 = 11.06$, $p<0.001$; Hp: Wald $X^2 = 19.60$, $p<0.001$). This effect was driven by females, who showed significantly greater activated caspase-3 immunoreactivity one week after hearing predators when compared to their non-predator counterparts in all three regions measured (LSD; PFC: $p<0.001$; Amg: $p=0.003$; Hp: $p<0.001$). Additionally, there was a significant difference between male and female voles in the non-predator controls in the amygdala (LSD $p=0.011$). In addition to the treatment x sex interactions, there was a significant main effect of treatment in the hippocampus (Wald $X^2 = 6.43$, $p=0.011$). There were no main effects of treatment in the prefrontal cortex (Wald $X^2 = 3.64$, $p=0.056$) or amygdala (Wald $X^2 = 0.8$, $p=0.4$), and no main effect of sex in any of the regions measured (all $p>0.7$).

4.4 Discussion

My results demonstrate the first experimental evidence that fear has PTSD-like, enduring effects on the brain and behaviour in free-living wildlife. I found that hearing the sounds of predators had enduring effects on nocturnal activity consistent with an elevated predation risk and increased dendritic length and branching in the basolateral amygdala.

For all immunohistochemistry markers quantified, I found that animals responded differently to treatment depending on their sex. In the prefrontal cortex, males increased neurogenesis (DCX) after hearing predator calls, compared to both the control males and females in the predator treatment. Males also had significantly greater activation (Δ FosB) in the prefrontal cortex than females after hearing predator calls. I also demonstrate enduring effects on apoptosis (activated caspase-3) in all areas of the brain sampled, wherein females showed increased apoptosis one week after hearing predator calls.

Voles demonstrated an enduring behavioural response to fear through altered nocturnal activity, where those who heard predator calls one week earlier were caught later in the night than those in the non-predator control treatment. Rodents have been shown to preferentially forage in darker environments when given the choice, and have a higher giving up density when foraging in illuminated environments (Farnworth et al. 2016, Zhang et al. 2020, Hoffmann et al. 2022). Given that my traps were set a couple hours before sunset, it is possible that animals in the predator treatment delayed nocturnal activity to take advantage of the increased perception of safety under increased darkness. Wild deer mice (*Peromyscus maniculatus*) have also been demonstrated to alter their activity, resulting in changes to capture time in live traps, between open and shrub covered habitats to minimize their risk of predation (Connolly and Orrock 2018). It is also possible that the voles were just less active overall in the predator treatment, and therefore less likely to encounter and enter a trap, as bank voles (*Myodes glareolus*) have shown lower levels of activity and consumed less food when in foraging in a habitat that was perceived to be higher risk due to increased light and lack of protective cover (Ferreira et al. 2023).

Predator induced fear had an enduring effect on neuron structure, where I found longer dendrites in the amygdala that was consistent across both sexes. This is consistent with laboratory animal models, where longer neurons have been reported in the basolateral amygdala in response to a stressful stimuli including predator cues and immobilization (Vyas et al. 2004, Cohen et al. 2014, Chattarji et al. 2015, Arluk et al. 2022). This increased dendritic length was linked to elevated anxiety-like behaviour that

persisted for at least 21 days after the termination of the stressful stimuli (Vyas et al. 2004), which suggests that the effects of predation risk on dendritic morphology are persistent and potentially permanent changes in the neuron structure. Anxiety-like behaviour was measured in the lab by a reduction in the number of entries to and time spent in the open arms of the elevated plus maze (Vyas et al. 2004), and represents an altered level of activity in stressed rodents. While it was not feasible to test wild voles in an elevated plus maze in the field, the time of entry to the live traps suggests that the voles who heard predator calls altered their nocturnal activity, which would suggest that my results are consistent with this previous laboratory experiment for both dendritic morphology in the amygdala and behaviour. In addition to increased anxiety behaviour, the amygdala has been shown to have an essential role in stress-induced facilitation of aversive learning and has been connected to stronger fear memories (Chattarji et al. 2015).

One common pattern across my measures of neurogenesis, activation, and apoptosis is that wherever I observed enduring effects of predator-induced fear, I found a treatment by sex interaction as males and females differ in both the magnitude and direction of their responses. While these enduring effects are interesting of their own accord, the reported sex differences are particularly interesting due to the lack of available data for female subjects in animal models for PTSD (Deslauriers et al. 2018). Sex differences in behavioural responses to stress have traditionally been viewed as differences in cognitive flexibility, often implying impairments in the females, while recent work has suggested that they may actually represent sex differences in survival strategies (reviewed in Shansky 2018). Females rats have been shown to be more risk averse than males, selecting the safer, less rewarding food option while males were more likely to select a larger, riskier reward (Orsini et al. 2016). This variation in survival strategies can also be seen in captive voles, where male showed a significant decrease in movement in response to predator odour, where females show significantly lower movements overall than males, matching the levels seen in males experiencing predator odour, but with no effect of predator odour (Perrot-Sinal et al. 1996).

When looking at the enduring effects of fear on neurogenesis, males in the predator treatment showed significantly greater DCX immunoreactivity in the prefrontal cortex compared to both males in the non-predator treatment and females in the predator treatment. Increased hippocampal neurogenesis is associated with the formation of new memories, often at the expense of older, established hippocampus-dependent memories (Frankland et al. 2013, Akers et al. 2014). While mammalian neurogenesis primarily occurs in the hippocampus, there is some evidence for neurogenesis, labelled with BrdU, occurring in the rodent neocortex (Gould 2007). Doublecortin expression, however, is generally associated with migrating neuronal precursors (Couillard-Despres et al. 2005). The prefrontal cortex is thought to be particularly important for the consolidation of fear memories after learning, where interference with plasticity in the prefrontal cortex after learning led to retention deficits 24 h and 48 h later (Euston et al. 2012). This would suggest that the increased DCX immunoreactivity in the prefrontal cortex of male voles after experiencing predator playbacks may be linked to the consolidation of fear memories through the incorporation of new neurons.

Voles who heard predators one week earlier had a significant sex difference in activation, with males showing greater Δ FosB immunoreactivity than their female counterparts, while there was no sex difference in activation in the non-predator controls. This follows the same pattern as seen in the hippocampus for DCX, which is consistent with guinea pigs who showed high levels of co-labelling between DCX and c-fos, a transcription factor from the same FOS family as Δ FosB, in response to increased environmental enrichment (Fan et al. 2014). The prefrontal cortex is predominantly important for the expression of older memories, with increased activation in the prefrontal cortex demonstrated after remote, but not recent, memory recall in mice (Frankland et al. 2004), suggesting that the increased Δ FosB activation seen in the male voles may be linked to recall of remote fear memories. Given that memories are considered remote after at least 12 days in rodents (Dixsaut and Gräff 2021), memories formed in the first 6 days of the playback manipulation would be considered remote and memories formed on the first day of playbacks would have been considered remote for 6 days of potential memory recall by the time brains were collected for analysis. While there is not a statistically significant treatment effect, male voles show the same pattern in

the increased Δ FosB found in prefrontal cortex of male experiencing social defeat stress (Santos-Costa et al. 2021). Increased activation in the prefrontal cortex in males has been linked to increased fear discrimination, while the lower levels of activation observed in females was linked to increased fear generalization (Day et al. 2020). Fear generalization may be beneficial for the females as they care for the offspring so being risk averse may promote offspring survival (McGuire and Novak 1984) while fear discrimination may be more beneficial for males to maximize dispersal and mate encounters in this promiscuous species (Boonstra et al. 1993).

In contrast to the male-driven enduring effects on neurogenesis, I found enduring effects of fear on apoptosis in the female voles for each brain region analyzed. Increased apoptosis is consistent with laboratory animal models, where increasing levels of activated caspase-3 have been reported in the prefrontal cortex, amygdala, and hippocampus lasting at least 7 days after single prolonged stress models of PTSD (Xiao et al. 2011, Yu et al. 2014, Han et al. 2021). Given that activated caspase-3 peaks between 2-4 hours after the initiation of apoptosis (Elliott and Ravichandran 2016), this suggests fear induces a prolonged elevation of apoptosis in brain regions associated with fear processing. Increased apoptosis in the hippocampus has been demonstrated in rats in conjunction with impaired extinction of fear memory after experiencing single prolonged stress (Araki et al. 2020), while increased apoptosis in the prefrontal cortex has been shown in rats with impaired learning and memory formation after experiencing single prolonged stress (Wen et al. 2017). This suggests that the increased apoptosis demonstrated in the female rats may be prolonging the retention of existing fear memories while also impairing their ability to form new memories and is consistent with PTSD-like behaviour.

Here I present the first experimental evidence that free-living wildlife demonstrate enduring, PTSD-like changes in neurobiology in response to predator-induced fear, consistent with an enduring memory of fear. Finding enduring effects in free-living wildlife supports the notion that PTSD-like enduring effects are evolutionarily adaptive, where retaining an enduring memory of previous predator encounters helps individuals survive future encounters and increases the likelihood of producing future offspring

(Clinchy et al. 2011). Demonstrating enduring effects of fear in free-living animals also provides ecological validity to laboratory animal models of PTSD by removing any potential confounds of captivity (Clinchy et al. 2011). This also addresses the concern that naïve, laboratory bred rodents may respond differently to predation risk than their wild counterparts, who likely have prior experience encountering predators or their cues (Clinchy et al. 2011, Cohen et al. 2023). I report enduring effects on dendritic morphology in both sexes, comparable to those found in males the laboratory (Vyas et al. 2004), and consistent with the notion that the enduring effects of PTSD may result from a hyperfunctional, rather than a dysfunctional, amygdala (Diamond and Zoladz 2016). Fear induced effects on neurogenesis are typically measured in the hippocampus, with suppression of neurogenesis reported (Tanapat et al. 2001, Vignisse et al. 2017), however I report an increase in immature neurons in the male prefrontal cortex in response to predator induced fear. Laboratory animal models demonstrate that both males and females increase Δ FosB in the prefrontal cortex in response to fear or chronic stress (Gerrits et al. 2006, Mackenzie et al. 2010, Ahlbrand et al. 2024). In my wild voles, male Δ FosB responds in the expected direction while females show the opposite effect, leading to the sex difference in my predator treatment. The enduring increase in apoptosis I see in females is consistent with laboratory models for PTSD in all three regions for male rodents (Xiao et al. 2011, Yu et al. 2014, Han et al. 2021) and in females in the hippocampus (Yakhkeshi et al. 2022, Mirjalili et al. 2022), while this is the first experiment to quantify PTSD-like effects on activated caspase-3 in the amygdala and prefrontal cortex of female rodents. In contrast, free living males, showed no enduring effect of fear on apoptosis. Overall, I demonstrate that while free-living animals have enduring effects on the same markers that are used to model PTSD in laboratory animals, the direction of effects or the brain region implicated may vary from the lab models. Demonstrating PTSD-like enduring effects in free-living wildlife supports the notion that PTSD-like effects are present in nature and that retaining a memory of fear may have an evolutionarily adaptive advantage, which can aide in reducing social stigmas for humans suffering from PTSD.

Table 4.1: List of species used in the auditory playbacks for the voles. Predator and non-predator species were matched based on their acoustic call characteristics (frequency and maximum amplitude).

Time	Predator	Non-predator
Day	Barred Owl (<i>Strix varia</i>)	European Starling (<i>Sturnus vulgaris</i>)
Day	Northern Harrier (<i>Circus cyaneus</i>)	Common Loon (<i>Gavia immer</i>)
Day	Red-shouldered Hawk (<i>Buteo lineatus</i>)	American Robin (<i>Turdus migratorius</i>)
Day	Red-tailed Hawk (<i>Buteo jamaicensis</i>)	Yellow-rumped Warbler (<i>Setophaga coronate</i>)
Night	Eastern Screech-Owl (<i>Megascops asio</i>)	Common Loon (<i>Gavia immer</i>)
Night	Saw-whet Owl (<i>Aegolius acadicus</i>)	Wood Frog (<i>Lithobates sylvaticus</i>)
Night	Barred Owl (<i>Strix varia</i>)	Leopard Frog (<i>Lithobates pipiens</i>)

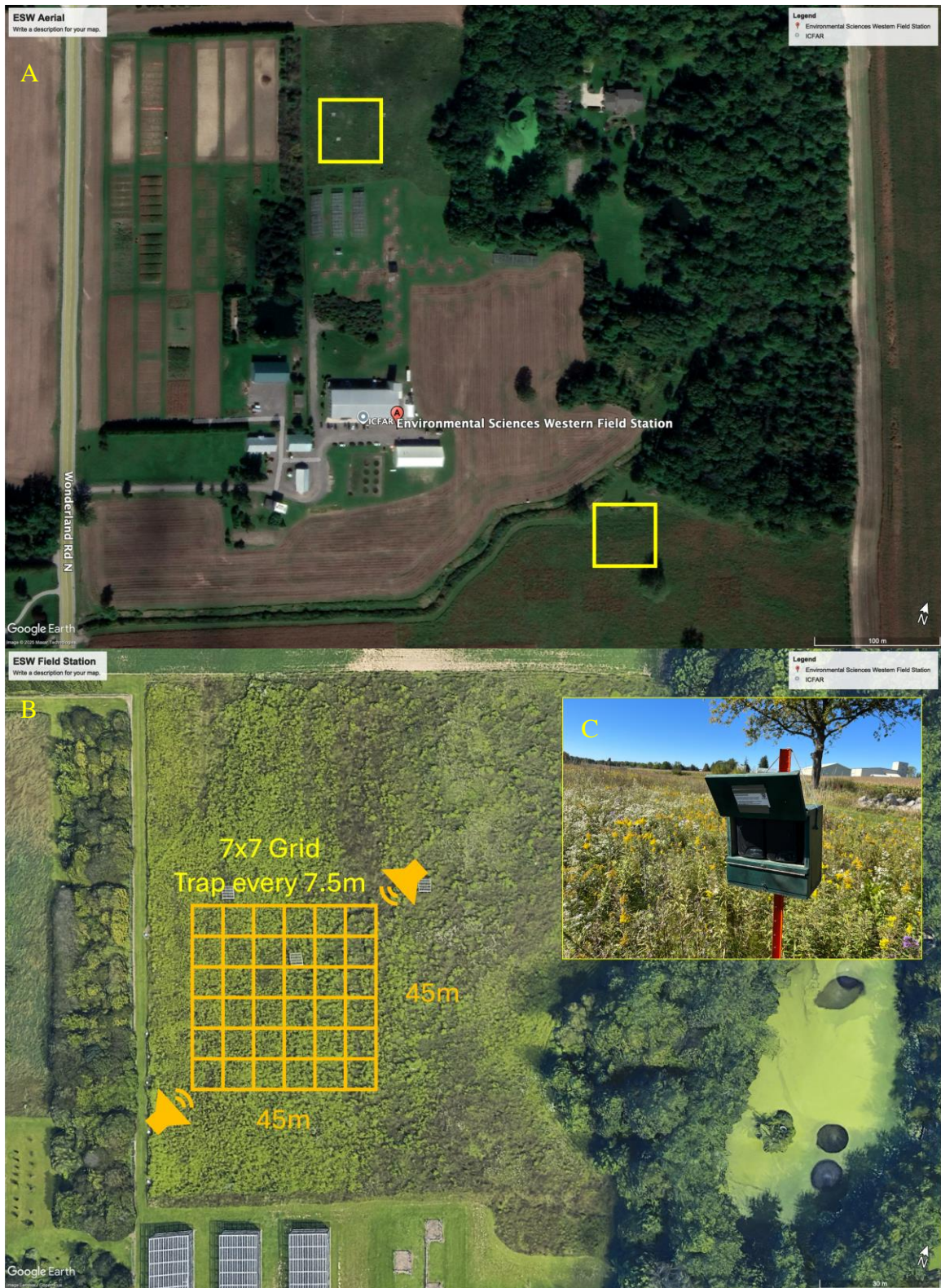


Figure 4.1: A) Aerial view of the grid setup at the ESW and B) schematic of the experimental grid setup at ESW with C) an inlay image of the speakers used in the field.

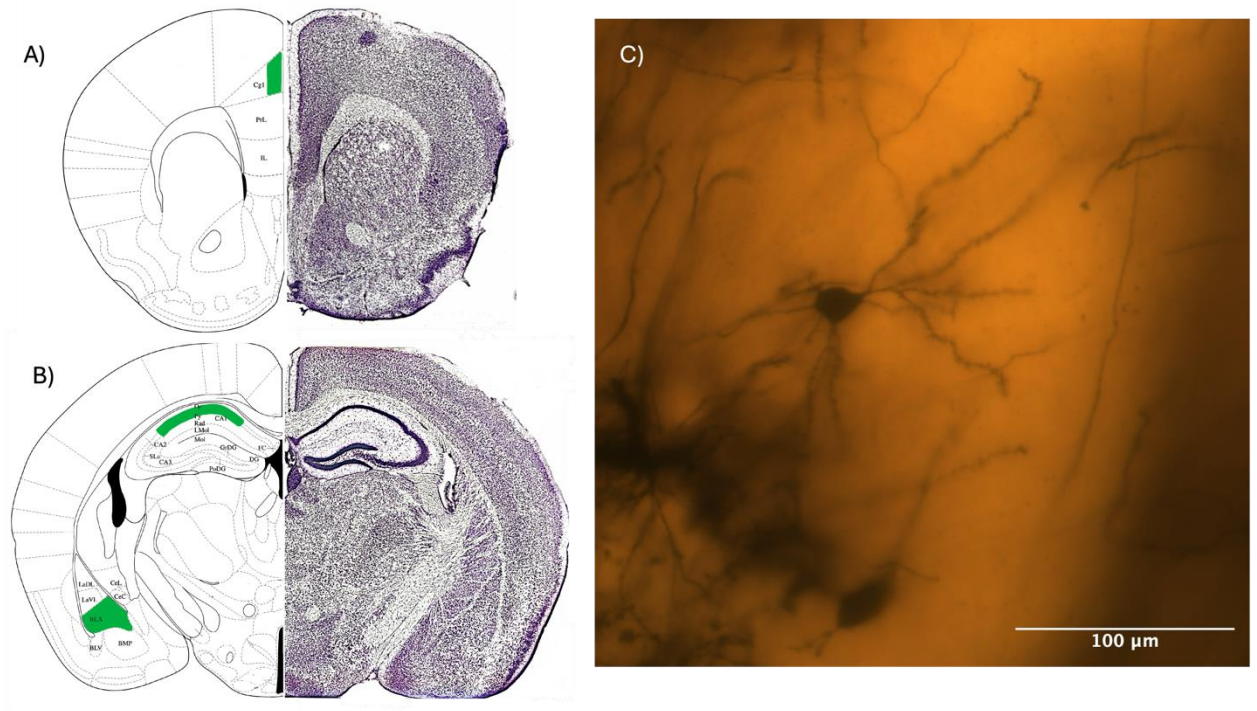


Figure 4.2: Brain areas photographed for dendritic morphology in the A) prefrontal cortex, B) amygdala, and hippocampus, with the subregions photographed labelled in green. Images are adapted from Paxinos and Franklin (Paxinos and Franklin 2013, 2019). Example photomicrograph of Golgi-Cox staining in the basolateral amygdala

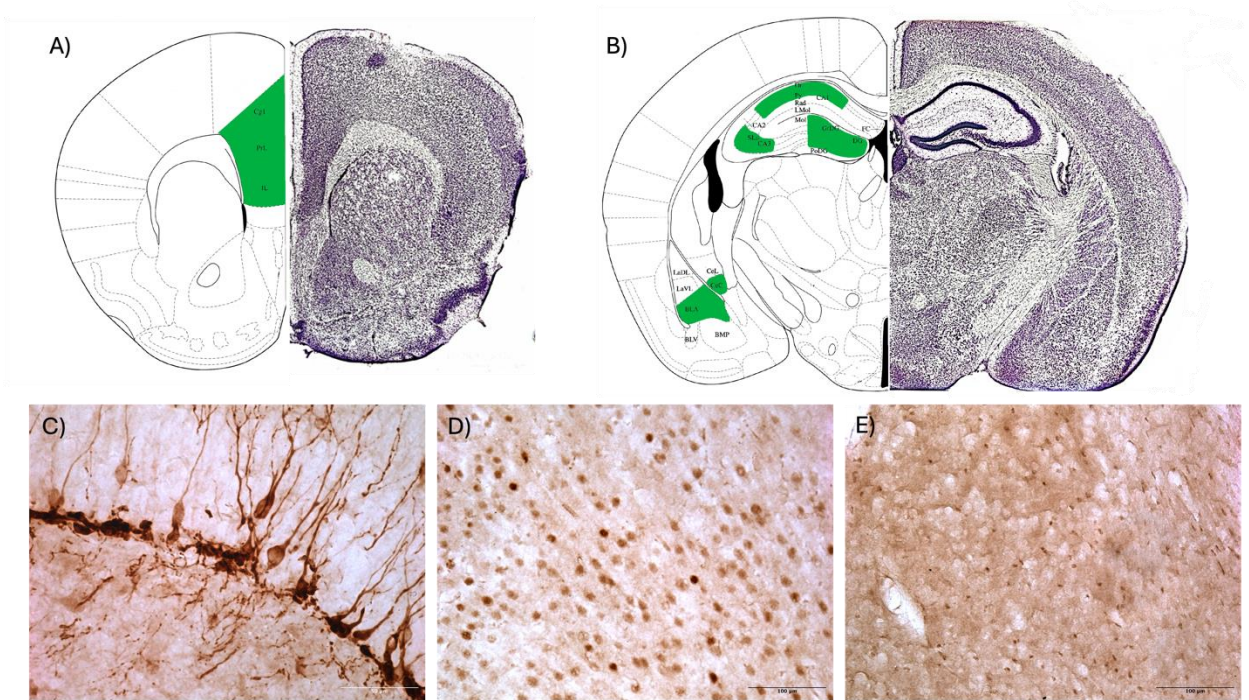


Figure 4.3: Brain areas photographed for immunohistochemistry in the A) prefrontal cortex, B) amygdala, and hippocampus, with the subregions photographed labelled in green. Images are adapted from Paxinos and Franklin (Paxinos and Franklin 2013, 2019). Example photomicrographs of staining for C) DCX in the dentate gyrus, D) Δ FosB in the cingulate cortex, E) activated caspase-3 in the basolateral amygdala.

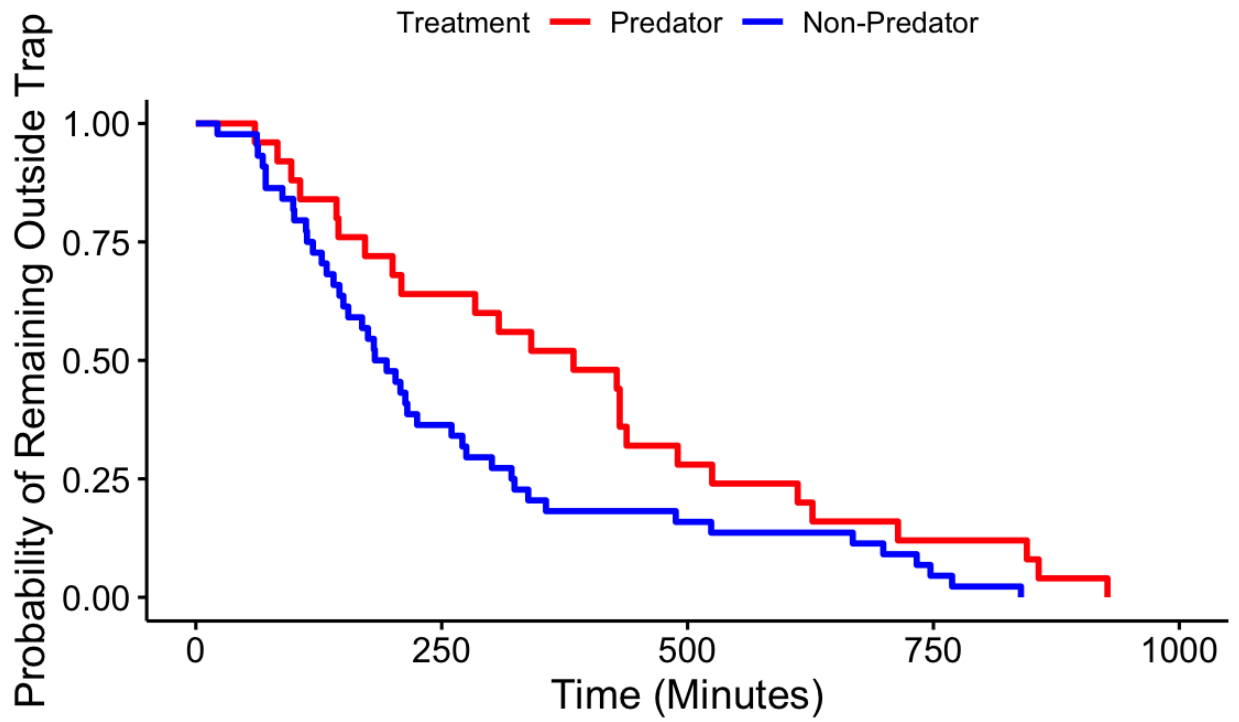


Figure 4.4: Kaplan-Meier survival curve from trap opening at 17:00 until time of entry to the Longworth live traps. N=69 voles (NP=44; Pred=25).

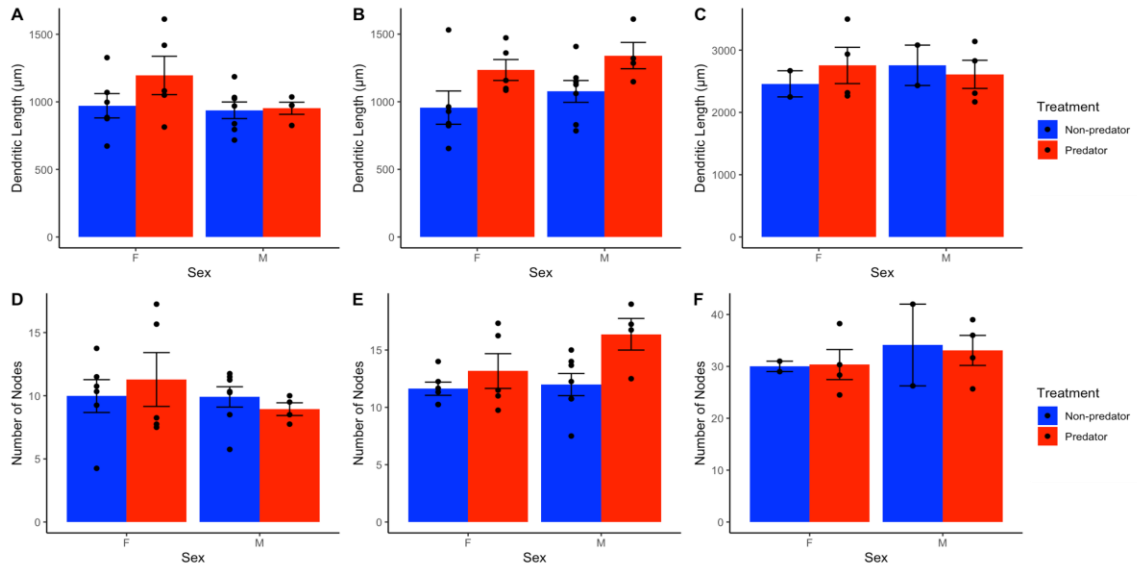


Figure 4.5: The total length of dendrites in A) prefrontal cortex, B) basolateral amygdala, and C) hippocampus and the number of branch nodes in the D) prefrontal cortex, E) basolateral amygdala and F) hippocampus. N= 22 voles (NP: 6F, 7M; Pred: 5F, 4M). Bars indicate means \pm standard error, filled circles indicate individual data.

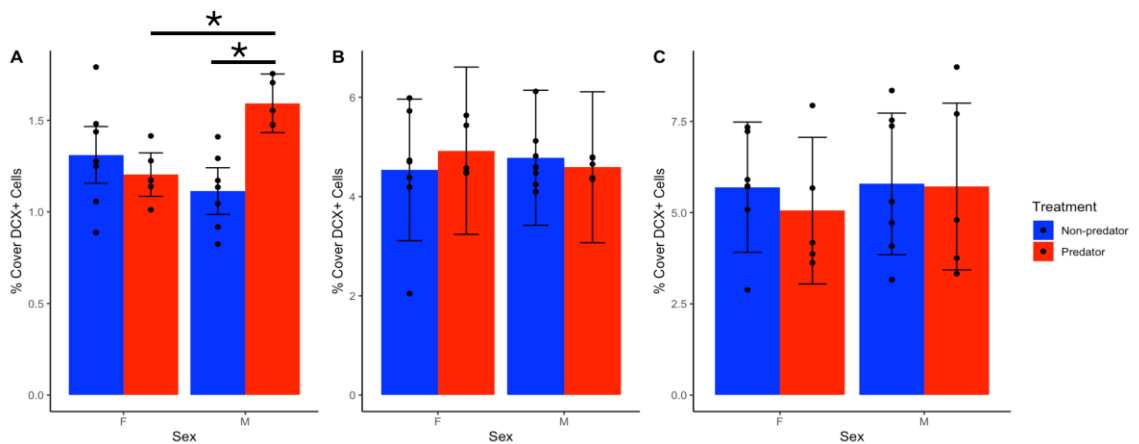


Figure 4.6: Neurogenesis: The effect of predator induced fear on the percent cover of DCX-ir cells in the A) prefrontal cortex, B) the amygdala, and C) the hippocampus. N= 24 voles (NP: 7F, 7M; Pred: 5F, 5M). Bars indicate means \pm standard error, filled circles indicate individual data.

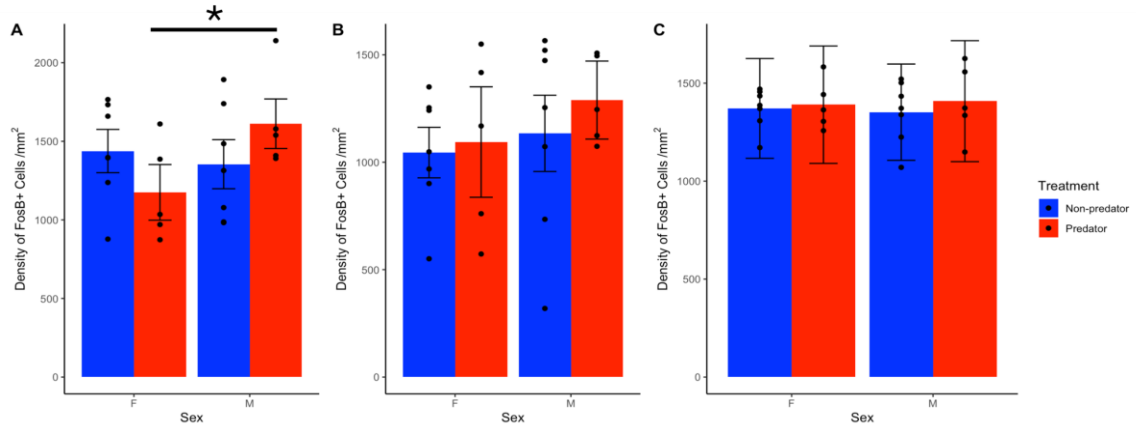


Figure 4.7: Activation: The effect of predator induced fear on the density of Δ FosB-ir cells in the A) prefrontal cortex, B) the amygdala, and C) the hippocampus. N= 24 voles (NP: 7F, 7M; Pred: 5F, 5M). Bars indicate means \pm standard error, filled circles indicate individual data.

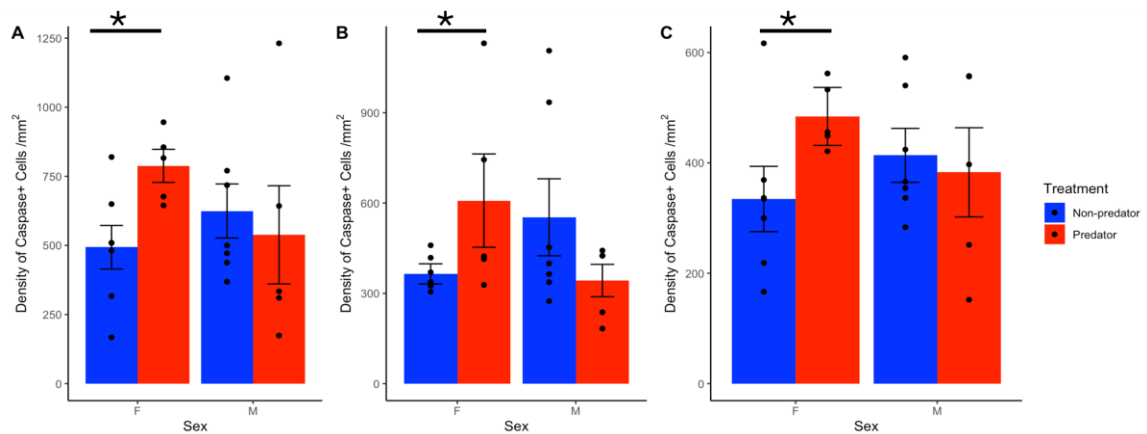


Figure 4.8: Apoptosis: The effect of predator induced fear on the density of activated caspase-3-ir cells in the A) prefrontal cortex, B) the amygdala, and C) the hippocampus. N= 24 voles (NP: 7F, 7M; Pred: 5F, 5M). Bars indicate means \pm standard error, filled circles indicate individual data.

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Chapter 5

5 General Discussion

5.1 Comparisons between fear manipulations in captive, semi-natural, and free-living animals

My research, both in this thesis and previously completed work, addressed the enduring effects of fear on the brain and behaviour in wild animals across multiple species and under increasingly natural conditions. Here, I have demonstrated that wild animals do show enduring effects of fear when held in captivity in the laboratory (Zanette et al. 2019, Chapter 2), when living semi-natural conditions in large outdoor aviaries (Witterick 2017, Chapter 3), and in free-living animals without any influence of captivity (Chapter 4). However, these enduring effects did not always present in the same manner, with variation between experiments in the brain regions where enduring effects presented, the direction of the effects, whether sex differences were found, and which markers showed the greatest change in the brain in response to predator induced fear (Figure 5.1).

Dendritic morphology was the most consistently affected neural measure across experiments for showing enduring effects in response to predator induced fear under semi-natural and free-living conditions. When quantifying dendritic morphology, I consistently found that where there were significant enduring effects, animals had longer dendrites and in most cases this also corresponded with increased dendritic branching. I demonstrate this in Chapter 3, where black-capped chickadees (hereafter chickadees) had longer neurons in the hippocampus (Hp) and caudolateral nidopallium (NCL), along with more branched NCL neurons one week after hearing predator calls in semi-natural conditions in the fall. In a comparable experiment on brown-headed cowbirds (hereafter cowbirds), I had previously demonstrated longer and more branched neurons in the Hp of females though males were not affected (Witterick 2017). In my current research, I further detected significant changes in the neurons of free-living female and male meadow voles (hereafter voles), who had longer neurons in the basolateral amygdala (Chapter 4) one week after hearing predator compared with non-predator vocalizations.

While there is some variation between experiments in the brain region exhibiting these enduring effects, it is clear that predator-induced fear has measurable effects on dendritic morphology in the brains of wild animals regardless of whether they are in the lab, outside in enclosures, or completely free-living. In addition to being demonstrated across increasingly natural conditions, fear induced alterations to dendritic morphology are likely to endure past the 7-day period measured in my manipulations. Alterations to dendritic morphology in response to fear conditioning have been shown to persist for at least 5 weeks without interventions promoting fear extinction (Heinrichs et al. 2013), suggesting that fear-induced alterations to dendritic morphology may be more persistent than the other neural markers measured. Even when dendritic morphology does recover after chronic stress, the new growth occurs on different dendrites than those that atrophied, leading to long lasting changes to the dendritic architecture, with the likelihood of recovering from stress induced alterations to dendritic morphology decreasing with age (McEwen and Morrison 2013). Finally, the fact that enduring effects of fear on dendritic morphology can be demonstrated in both birds and mammals suggests that this is a reliable marker of fear induced changes in the brain and likely to be present in other taxa.

While dendritic morphology carried a signature of the enduring effects of fear across manipulations, species and taxonomic group, I found more variation in responses to all immunohistochemistry assays. When quantifying the enduring effects of fear on Δ FosB on the brains in wild animals, there is more variation as to whether significant enduring effects are detected in the brain. Significantly increased Δ FosB was previously detected in both the Hp and medial ventral arcopallium (AMV) in chickadees after hearing predator calls under laboratory conditions (Zanette et al. 2019). I also show a treatment by sex interaction for Δ FosB in the prefrontal cortex (PFC) of free-living voles, with a significant difference in the response between males and females who previously heard predator calls (Chapter 4). In contrast, there was no effect on Δ FosB detected in either chickadees (Chapter 3) or in cowbirds (Witterick 2017) hearing predators in the large outdoor aviaries. Δ FosB is widely used as a marker of enduring activation and particularly implicated for its role in the reward circuitry of the brain and addiction

(Robison and Nestler 2022). Increased Δ FosB has also been demonstrated in response to predator cues in laboratory studies (Staples et al. 2009, Mackenzie et al. 2010). The lack of measurable enduring effects under semi-natural conditions in both the chickadees and the cowbirds, along with the voles demonstrating a difference in Δ FosB between males and females who heard predator calls but no significant treatment effect within either sex suggests that Δ FosB may be more reliably used as a marker of enduring fear effects in laboratory experiments, particularly for the avian brain. To date, there have only been three studies looking at Δ FosB in the avian brain in response to predators, and all have come from our lab (Witterick 2017, Zanette et al. 2019, Chapter 3). Additional research on the role of captivity on Δ FosB activation would also be beneficial to better understand the variability I have found between the laboratory, semi-natural, and free-living experiments when looking at the enduring effects of fear on the neurobiology.

Changes in neurogenesis in response to predator-induced fear were demonstrated by changes to immature neurons (DCX) in the AMV of chickadees in the laboratory (Chapter 2), in the Hp and AMV of cowbirds in semi-natural conditions in the spring (Witterick 2017), and in the PFC of free male living voles (Chapter 4), while I found no enduring effect on immature neurons in the brains of chickadees in semi-natural conditions (Chapter 3). The enduring effects of fear on cell proliferation (PCNA) were much more variable than they were for immature neurons (DCX). I reported suppression of PCNA in the Hp of males in the laboratory (Chapter 2), no enduring effect in the chickadees under semi-natural conditions (Chapter 3), and with effects varying by month in the cowbirds under semi-natural conditions (Witterick, unpublished data). Due to the variability in the PCNA results from the avian studies, I did not measure the effects of fear on PCNA in the voles. Suppressed neurogenesis in response to predator cues has been demonstrated in lab rodents for both cell proliferation (Tanapat et al. 1999, Vignisse et al. 2017) and immature neurons (Vignisse et al. 2017, Wu et al. 2019). Suppressed cell proliferation was also demonstrated in response to simulated predator attacks in fish (Dunlap et al. 2017). In contrast, in the male voles I found an increase in immature neurons in the PFC, indicating that more new neurons were migrating to the PFC after exposure to predator cues. Previous work co-labelling DCX with BrdU to determine cell

age suggests that DCX is a reliable marker of cells up to 30 days old, while still detected in lower quantities up to 60 days (Balthazart and Ball 2014). In contrast, the half-life of PCNA is 20 h in rodents (Bravo and Macdonald-Bravo 1987), presumably making it more susceptible to environmental variation experienced by wildlife. Overall, my results suggest that labelling immature neurons with DCX provides a more reliable marker of the enduring effects of fear on the neurogenesis than labelling cell proliferation with PCNA.

The enduring effects of predator-induced fear on apoptosis were only possible to measure in the free-living meadow voles (Chapter 4), because there is not currently a marker to consistently and reliably label apoptosis in the songbird brain (MacDougall-Shackleton et al., unpublished data). I found a treatment by sex interaction with female voles showing increased activated capase-3 in all three regions quantified. Increased apoptosis is consistent with animal models showing increased activated caspase-3 lasting at least 7 days after experiencing the single prolonged stress model for PTSD (Xiao et al. 2011, Yu et al. 2014, Han et al. 2021).

5.2 Novelty of Non-traditional study systems

Using wild caught animals to study the enduring effects of fear on the brain is a unique aspect of this work, as animal models for PTSD typically use laboratory strains of rats and mice (Deslauriers et al. 2018). While laboratory rodents have many benefits for this line of study due to the large body of available literature on fear effects, brain mapping, and ease of access for acquiring study animals, they also present some challenges in our ability to extrapolate enduring effects seen in animal models to other taxa, including humans. The first challenge is that animals are often bred for several tens of generations in captivity (Benavides and Jaubert 2024), making predator cues a novel experience in addition to the innate fear response. Wild caught animals, particularly those captured as adults, likely have prior experience with predators or their cues, making any effects on the brain measured in our experiments a result of heightened predator-induced fear rather than fear of a novel stressor. Laboratory bred rodents are also subject to many generations of inbreeding, reducing genetic variation (Brekke et al. 2018, Benavides and Jaubert 2024). While this can be advantageous by increasing experimental power and limiting noise due to genetic variation (Brekke et al. 2018), inbreeding can be also

amplify traits that would otherwise appear in very few individuals within a population due to founder effects or genetic drift (Brekke et al. 2018). In contrast, genetic analysis of wild voles living in geographically isolated populations still maintained some genetic diversity in the mitochondrial haplotypes measured despite evidence of founder effects (Francl et al. 2008). Finally, demonstrating comparable enduring effects of fear on the brain in new species, as I have shown in both chickadees and voles, suggest that these effects may be widespread across taxa and may provide avenues for further investigation into therapeutic treatments to reduce PTSD symptoms in humans.

Demonstrating enduring effects of fear on the brain in wildlife supports the notion that there may be evolutionary adaptive benefits to PTSD-like changes in the brain, if these changes the brain lead to enduring memories of fear that promote survival in future predator encounters (Cantor 2009, Clinchy et al. 2011, Diamond and Zoladz 2016, Cohen et al. 2023). Finding enduring, PTSD-like changes in the brain in wild animals after hearing the sounds of their natural predators also adds ecological validity to the predator models of PTSD used in the laboratory (Clinchy et al. 2011). Demonstrating enduring effects of fear on the brain in both birds and rodents, all of which were wild caught and likely had prior experience encountering predators or their cues, suggests that PTSD-like effects may be widespread in nature, with adaptive benefits to survival, and may help to reduce the stigma for humans afflicted with PTSD.

5.3 Limitations to this research

As is always the case with scientific research, my experiments did have potential limitations that I will address here. The first would be the sample size, particularly for Chapter 2. When working with wildlife it is necessary to balance the desire with a large sample size with the ethical requirement to minimize the number of animals used for research purposes. In particular for Chapter 2, the sample size was limited due to using tissue from animals that had been collected for an earlier study, so it was not feasible to add additional subjects as this would add additional experimental noise due to variation in the year of capture. In addition to the sample size, the number and geographic location of replicates, particularly for the aviary experiments, is another potential limitation of these experiments. Expanding to use additional aviaries, additional replicates within the

same aviaries, or working with aviaries where the simultaneous replicates were geographically farther apart would add additional statistical power and support for the replicability of these experiments. Another limitation is my ability to attribute any effects measured as solely due to the predator playbacks rather than a response to the non-predator controls. Future experiments could address this limitation by adding in an additional control with white noise or other audio that would control for the response to sound without conveying any additional information to the animals. Third, while I do attribute the enduring response in the brain as suggestive of an enduring memory of fear based on comparable changes in the brain in the laboratory animal model literature, additional cognitive behavioural testing would be required to distinguish whether these differences are solely due to memory or whether they are also in response to learning. Future research could address this question with the addition of cognitive behavioural testing prior to collection of the brains. Finally, the last limitation that I will address is the variation in previous life experience among the study animals. All animals used in my experiment were captured from the wild and therefore their life experience up until the point of capture, particularly their prior experience with predators, is unknown. The fact that I was able to detect differences between experimental treatments despite the expected variability in previous life experience and life history suggests that the effects measured are robust, but it is still an unknown factor that I was unable to control for. Looking at the effect of previous life experience on the neuronal response to predator cues could be an interesting avenue of future study to address this limitation.

5.4 Future Directions

I have demonstrated the enduring effects of fear on the brains of wild caught chickadees in both a laboratory environment and semi-natural conditions and in free-living voles. To better understand the ubiquity of these enduring effects, future research is needed to conduct predation risk manipulations in free-living animals of other taxa. Laboratory studies have shown that fear leads to enduring suppression of cell proliferation in electric fish (*Brachyhypopomus gauderio* and *Apteronotus leptorhynchus*; Dunlap et al. 2017), while correlational studies showed reduced cell proliferation in electric fish (*Brachyhypopomus occidentalis*) and Trinidadian killifish (*Rivulus hartii*) living in

naturally high predation environments compared to environments with few predators (Dunlap et al. 2016, 2019). Similar correlational studies have revealed altered brain anatomy, larger brains, and increased vasotocin gene expression in guppies (*Poecilia reticulata*) living in environments with naturally high predator pressure (Kotrschal et al. 2017, Reddon et al. 2018, 2022, Reyes et al. 2022). This suggests that manipulating perceived predation risk would lead to enduring effects on the brain in fish, but this has yet to be experimentally tested. It would also be beneficial to expand from laboratory and semi-natural studies to measure the effects of fear on the brain in free-living birds, as previous work has shown that fear alone can reduce wild song sparrow populations with intergenerational impacts (Zanette et al. 2011, Allen et al. 2022). Enduring effects that are consistent across taxa may also provide a more targeted approach to potential avenues of research assess therapeutic interventions for PTSD. While I have demonstrated that the fear of predator does have enduring, PTSD-like effects on neurobiology in free-living meadow voles, questions have been raised as to how the behavioural stress responses observed in the lab would translate to their wild counterparts (Cohen et al. 2023). Assessing potential therapeutic interventions for PTSD in wild rodent models could be an accessible and budget conscious intermediary between laboratory rodent models and human clinical research.

In my research I have demonstrated enduring effects of fear on the brain, focusing on the hippocampus, amygdala, and prefrontal cortex in mammals and the homologous and analogous counterparts in the avian brain. While these three brain regions are widely studied for their roles in the fear processing pathway, there are additional brain regions implicated in the fear processing pathway that could provide new avenues of research to widen our understanding of the enduring effects of fear on neurobiology. Two regions that are often mentioned for their roles in the fear processing pathway are the mammalian periaqueductal grey (PAG) and the hypothalamus (Gross and Canteras 2012). The PAG is suggested to play an important role in prey defensive behaviours, including the fight or flight response and freezing behaviours, with the function thought to be highly conserved across species (Deng et al. 2016, Franklin 2019). In the avian brain, it is the intercollicular nucleus and the central mesencephalic grey that are thought to be functionally comparable to the mammalian PAG (Dubbeldam and den Boer-Visser 2002,

Kingsbury et al. 2011). PAG activation has been shown to induce defensive behaviours such as running, freezing, and avoidance (Deng et al. 2016), while predator odour has increased PAG activation (Sukikara et al. 2010). The hypothalamus, part of the hypothalamic-pituitary-adrenal axis, is highly conserved across vertebrate species as an essential component for the regulation of physiological homeostasis and behaviour (Denver 2009, Xie and Dorsky 2017). The hypothalamus has been implicated as part of the pathway for processing predator cues (Gross and Canteras 2012), with predator scent increasing activation in mice (Janitzky et al. 2015). Both regions have been demonstrated to play important roles in the fear processing pathways in laboratory rodent studies, suggesting that these regions may be of interest when looking at the enduring effects of fear on the brain.

In addition to expanding to study other areas of the brain, it would be beneficial to explore the enduring effects of predator-induced fear in free living animals on other markers used in laboratory models for PTSD. One marker of interest is brain derived neurotrophic factor (BDNF), a neurotrophic factor involved in neuronal survival, differentiation, and plasticity (Bathina and Das 2015). BDNF has been demonstrated to play an important role in neurogenesis, synaptic plasticity, and fear memory consolidation and extinction (Bathina and Das 2015, Notaras and van den Buuse 2020, Mojtabavi et al. 2020). In humans, participants with PTSD were found to have higher blood BDNF concentrations than the control subjects (Mojtabavi et al. 2020). Given the links to both neurogenesis and synaptic plasticity, quantifying BDNF in brain regions associated with fear processing would be an interesting and logical next step towards understanding the enduring effects of fear on the brain. Another potential marker of interest for future study of predator-induced fear is epigenetic changes through DNA methylation. In particular, the methylation of glucocorticoid receptors, which has been implicated for potential involvement in the intergenerational transmission of PTSD (Bhattacharya et al. 2019). In humans, blood samples from military members undergoing PTSD treatment showed significant changes in DNA methylation across 12 different genes when compared to their counterparts in remission for PTSD (Vinkers et al. 2021). Animal models quantifying epigenetic changes in response to predator-induced fear could help to fill gaps in the literature, where few studies have invested epigenetic

variation in the human brain post mortem due to a lack of available tissue from individuals with a history of PTSD (Morrison et al. 2019).

Future research on the role of sex in the neural and behavioural response to predator-induced fear is necessary our understanding of the enduring effects of fear. In both Chapter 2 and Chapter 4, I presented sex differences in the neuronal response to fear for at least one marker. Female subjects, however, are underrepresented in the PTSD animal model literature, despite being more susceptible to the predator-stress model for PTSD (Deslauriers et al. 2018). Sexual dimorphism has been reported for fear conditioning and extinction, the neurocircuitry of fear, and PTSD heritability, with variation between the sexes linked to estrogen levels (Ramikie and Ressler 2018). This may be a particularly interesting direction of study in seasonally breeding animals, where sex hormones can fluctuate greatly between seasons (Lincoln and Short 1980), potentially leading to seasonal variation in the behavioural, physiological, and neuronal response to fear in free living animals. Further research into the effects of seasonality on the enduring response to predator induced fear may also provide insight the extent that varying environmental conditions interact with the enduring effects of fear on the brain and behaviour.

To understand the neural underpinnings of the demographic consequences of predator induced fear (Zanette et al. 2011, Allen et al. 2022), future research is needed on the intergenerational effects of fear on the brain and behaviour in wildlife. A recent laboratory experiment showed that when mice were exposed to a rat predator, their offspring showed increased hippocampal activity and anxiety-like behaviour in response to a mild stressor, with these behavioural phenotypes persisting in the grand-offspring when exposed to the same mild stressor (Bhattacharya et al. 2023). Predator induced fear has also been demonstrated to have intergenerational effects reducing population growth in free-living birds, with projections suggesting that the population would be halved within 5 years (Allen et al. 2022). Meadow voles would be an ideal study species to quantify intergenerational effects of fear on the brain in wildlife, due to their short (21d) gestation, high reproductive potential, and rapid growth to sexual maturity (Kurta 2017). Intergenerational effects of PTSD have also been observed in humans, but the

mechanisms involved are not fully understood (Bowers and Yehuda 2016, Yehuda and Lehrner 2018). Quantifying the intergenerational effects of predator induced fear on the brain and behaviour in free living wildlife could provide insight into the neural underpinnings of the demographic consequences of fear observed in free-living birds (Zanette et al. 2011, Allen et al. 2022) along with providing new animal models to investigate the mechanisms underlying the intergenerational effects of PTSD in humans (Bowers and Yehuda 2016, Yehuda and Lehrner 2018) and target potential therapeutic treatments for those afflicted.

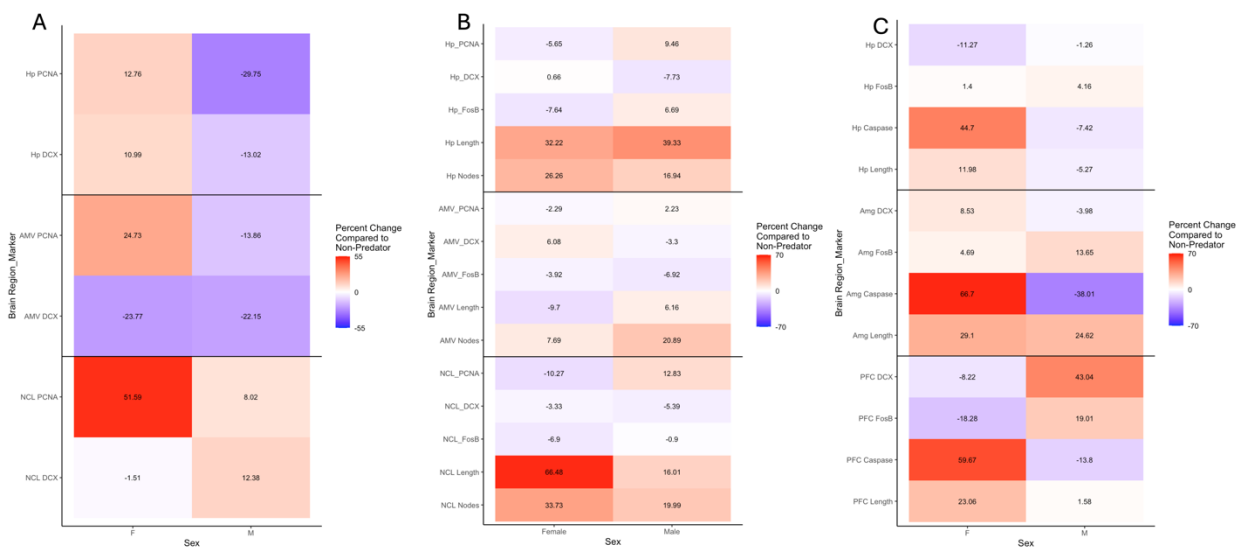


Figure 5.1: Heat maps displaying the percent change in the average value for each marker when compared to the non-predator control treatment, measured in females and males in A) the laboratory captive chickadees from Chapter 2, B) the chickadees housed in semi-natural aviaries from Chapter 3, and C) the free-living voles from Chapter 4. Hp: hippocampus; AMV: medial ventral arcopallium; NCL: caudolateral nidolpallium; Amg: amygdala; PFC: prefrontal cortex

5.5 References

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Appendices

Appendix A: Ethics approvals and permits



PI :	Zanette, Liana
Protocol #	2018-023
Status :	Approved (w/o Stipulation)
Approved :	06/01/2018
Expires :	06/01/2022
Title :	The biology of predator-induced fear: from brains to population to ecosystems



AUP Number: 2022-080

PI Name: Zanette, Liana

AUP Title: The biology of predator-induced fear: from brains to population to ecosystems. Deermice

Approval Date: 09/01/2022

Official Notice of Animal Care Committee (ACC) Approval:

Your new Animal Use Protocol (AUP) 2022-080:1: entitled " The biology of predator-induced fear: from brains to population to ecosystems. Deermice" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
 - o [Western's Senate MAPP 7.12 \[PDF\]](#); and
 - o [Applicable Animal Care Committee policies and procedures](#).
2. Prior to initiating any study-related activities—[as per institutional OH&S policies](#)—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
 - o Completed the appropriate institutional OH&S training;
 - o Completed the appropriate facility-level training; and
 - o Reviewed related (M)SDS Sheets.

Submitted by: Copeman, Laura on behalf of the Animal Care Committee



Environment and
Climate Change Canada

Environnement et
Changement climatique Canada

Scientific Permit
issued under sections 4 and 19 of the *Migratory Birds Regulations*,
C.R.C. c.1035 pursuant to section 12 of the *Migratory Birds*
Convention Act, 1994, S.C. 1994, c.22

Permit number:

SC-OR-2019-00044

Valid From: (yyyy/mm/dd)

2019/07/05

Expiry date: (yyyy/mm/dd)

2022/08/31

Report due annually on: (mm/dd)

01/31

Permit holder:

Liana Zanette
University of Western Ontario

Address:

████████████████████
████████████████████
████████████████████

Telephone number

██████████████

Email:

████████████████████

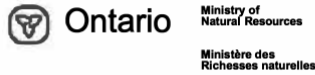
Under this permit, the permit holder is authorized to:

Activities	Species	Applicable locations
Kill	Black-capped Chickadee (<i>Poecile atricapillus</i>)	1
Possession	Black-capped Chickadee (<i>Poecile atricapillus</i>)	1
Take	Black-capped Chickadee (<i>Poecile atricapillus</i>)	1

Location(s):

1. London, Ontario (London area, around University of Western Ontario, UWO properties in Middlesex County)

General Terms and Conditions:



Wildlife Scientific Collector's Authorization
Autorisation pour faire la collecte scientifique d'animaux sauvages

Authorization No. N° d'autorisation	1101085
Local Reference No. N° de référence local	00175
Issuer Account No. N° de compte du délivreur de permis.	10003168

This authorization is issued under Section 39 of the Fish and Wildlife Conservation Act, 1997 to:
 Cette autorisation est délivrée en vertu de l'article 39 de la Loi sur la protection du poisson et de la faune de 1997 à:

Name of Authorization holder	Last Name / Nom de famille Ms. Zanette	First Name / Prénom Liana	Middle Name / Second Prénom
Nom du titulaire de l'autorisation	Name of Business/Organization/Affiliation (if applicable) Nom de l'entreprise/de l'organisme/de l'affiliation (le cas échéant) University of Western Ontario		

Mailing address of Authorization holder	Street Name & No./PO Box/RRW/Gen. Del./ N° rue/C.P./R.R./poste restante		
Adresse postale du titulaire de l'autorisation	City/Town/Municipality / Ville/village/municipalité London	Province/State Province/Etat ON	Postal Code/Zip Code Code Postal/Zip [REDACTED]

This authorization permits the above-named person to:
 Cette autorisation permet à la personne nommée ci-haut de:

- Capture wildlife of the species and sex, in the numbers, and in the area set out below.
Capturer les espèces d'animaux sauvages selon le nombre et le sexe indiqués ci-dessous dans les lieux indiqués ci-dessous and/or / et/ou
- Keep game wildlife or specially protected wildlife in captivity for the purposes of education or science.
Garder des animaux sauvages spécialement protégés et du gibier sauvage en captivité à des fins éducatives et scientifiques
- Release the captured wildlife in the area of capture, if the captured wildlife is not to be removed from that area
Remettre en liberté les animaux sauvages capturés dans la zone de capture si les animaux capturés ne doivent pas être enlevés de cette zone

OR / OU

- Capture and kill wildlife of the species and sex, in the numbers, and in the area set out below.
Capturer et tuer les espèces d'animaux sauvages selon le nombre et le sexe indiqués ci-dessous dans les lieux indiqués ci-dessous

Species / Espèces	Sex Sexe	Numbers Nombre	Location / Endroit
White-footed Mouse		120	Rare Charitable Research Reserve, 1679 Blaire Road Cambridge
Meadow Vole		120	Rare Charitable Research Reserve, 1679 Blaire Road Cambridge

Yes/Oui Additional list attached / Liste additionnelle ci-jointe

Authorization Dates / Dates d'autorisation	Effective Date / Date d'entrée en vigueur (YYYY-MM-DD) 2022-08-26	Expiry Date / Date d'expiration (YYYY-MM-DD) 2022-12-31
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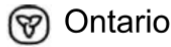
This authorization is subject to the conditions contained in Schedule A if included./Cette autorisation doit respecter les conditions de l'annexe A si celle-ci est jointe.

Conditions de l'autorisation	Yes/Oui <input checked="" type="checkbox"/> No/Non <input type="checkbox"/>	Schedule A included. / Annexe A ci-jointe
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Authorized by (please print) Autorisé par (veuillez écrire en caractères d'imprimerie) Deanna Lindblad - District Supervisor	Signature of Authorizer / Signature de la personne chargée d'autoriser [REDACTED]	Date of Issue/Date de délivrance (YYYY-MM-DD) 2022-08-26
Signature of Authorization holder / Signature du titulaire de l'autorisation [REDACTED]	Date (YYYY-MM-DD) 2022-08-26	

Personal information contained on this form is collected under the authority of the Fish and Wildlife Conservation Act, 1997 and will be used for the purpose of licensing, identification, enforcement, resource management and customer service surveys. Please direct further inquiries to the District Manager of the MNR issuing district.

Les renseignements personnels dans ce formulaire sont recueillis conformément à la Loi sur la protection du poisson et de la faune, 1997, et ils seront utilisés aux fins de délivrance de permis, d'identification, d'application des règlements, de gestion des ressources et de sondage sur les services à la clientèle. Veuillez communiquer avec le chef du district du MRN qui délivre le permis si vous avez des questions.



Ministry of
Natural Resources
Ministère des
Richesses naturelles

Wildlife Scientific Collector's Authorization

Autorisation pour faire la collecte scientifique d'animaux sauvages

Authorization No. N° d'autorisation	1102124
Local Reference No. N° de référence local	2022
Issuer Account No. N° de compte du délivreur de permis.	10003042

This authorization is issued under Section 39 of the Fish and Wildlife Conservation Act, 1997 to:

Cette autorisation est délivrée en vertu de l'article 39 de la Loi sur la protection du poisson et de la faune de 1997 à:

Name of Authorization holder	Last Name / Nom de famille Ms. Zanette	First Name / Prénom Liana	Middle Name / Second Prénom
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Nom du titulaire de l'autorisation
Name of Business/Organization/Affiliation (if applicable)
Nom de l'entreprise/de l'organisme/de l'affiliation (le cas échéant)

University of Western Ontario

Mailing address of Authorization holder	Street Name & No./PO Box/RR#/Gen. Del./N° rue/C.P./R./R./poste restante		
Adresse postale du titulaire de l'autorisation	City/Town/Municipality / Ville/village/municipalité London	Province/State Province/État ON	Postal Code/Zip Code Code Postal/Zip

This authorization permits the above-named person to:

Cette autorisation permet à la personne nommée ci-haut de:

- Capture wildlife of the species and sex, in the numbers, and in the area set out below.
Capturer les espèces d'animaux sauvages selon le nombre et le sexe indiqués ci-dessous dans les lieux indiqués ci-dessous and/or / et/ou
- Keep game wildlife or specially protected wildlife in captivity for the purposes of education or science.
Garder des animaux sauvages spécialement protégés et du gibier sauvage en captivité à des fins éducatives et scientifiques
- Release the captured wildlife in the area of capture, if the captured wildlife is not to be removed from that area.
Remettre en liberté les animaux sauvages capturés dans la zone de capture si les animaux capturés ne doivent pas être enlevés de cette zone

OR / OU

- Capture and kill wildlife of the species and sex, in the numbers, and in the area set out below.
Capturer et tuer les espèces d'animaux sauvages selon le nombre et le sexe indiqués ci-dessous dans les lieux indiqués ci-dessous

Species / Espèces	Sex Sexe	Numbers Nombre	Location / Endroit
Meadow Vole (to be released)		120	Middlesex County, City of London
Meadow Vole (to be euthanized)		40	Middlesex County, City of London

Yes/Oui

Additional list attached / Liste additionnelle ci-jointe

Authorization Dates Dates d'autorisation	Effective Date / Date d'entrée en vigueur (YYYY-MM-DD) 2022-10-12	Expiry Date / Date d'expiration (YYYY-MM-DD) 2022-12-31
--	---	---

Authorization conditions This authorization is subject to the conditions contained in Schedule A if included./Cette autorisation doit respecter les conditions de l'annexe A si celle-ci est jointe.

Conditions de l'autorisation
Yes/Oui No/Non Schedule A included. / Annexe A ci-jointe

Authorized by (please print) Autorisé par (veuillez écrire en caractères d'imprimerie) Deanna Lindblad	Signature of Authorizer / Signature de la personne chargée d'autoriser 	Date of Issue/Date de délivrance (YYYY-MM-DD) 2022-10-12
--	--	--

Signature of Authorization holder / Signature du titulaire de l'autorisation 	Date (YYYY-MM-DD) 2022-10-12
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Personal information contained on this form is collected under the authority of the Fish and Wildlife Conservation Act, 1997 and will be used for the purpose of licensing, identification, enforcement, resource management and customer service surveys. Please direct further inquiries to the District Manager of the MNR issuing district.

Les renseignements personnels dans ce formulaire sont recueillis conformément à la Loi sur la protection du poisson et de la faune, 1997, et ils seront utilisés aux fins de délivrance de permis, d'identification, d'application des règlements, de gestion des ressources et de sondage sur les services à la clientèle. Veuillez communiquer avec le chef du district du MRN qui délivre le permis si vous avez des questions.

Curriculum Vitae

Name: Lauren Witterick

Post-secondary Education and Degrees: Queen's University
Kingston, Ontario, Canada
2010-2014 B.Sc. (Hons)

The University of Western Ontario
London, Ontario, Canada
2015-2017 M.Sc.

The University of Western Ontario
London, Ontario, Canada
2017-2025 Ph.D.

Honours and Awards: Robert and Ruth Lumsden Graduate Award, Western University
2024

Third Place, Fallona Family Interdisciplinary Science Award,
Western University
2024

Karen Auzins Award, Western University Biology
2023

Runner-up Best Talk in Ethology, Ontario Ecology, Ethology, &
Evolution Colloquium
2023

Ages Foundation Fellowship, rare Charitable Research Reserve
2022

Ontario Graduate Scholarship, QEII/GSST
2020-2021

Western Graduate Research Scholarship
2015-2021

Ontario Graduate Scholarship
2018-2020

Biology Travel Award, Western University
2018

Ontario Graduate Scholarship, QEII/GSST
2017-2018

Biology Travel Award, Western University
2017

Scottish Rite Charitable Foundation Research Bursary
2017

Mary C. Holdsworth Scholarship
2015-2023

Queen's University Dean's Honour List
2014

Mary C. Holdsworth Scholarship
2010-2013

Queen's University Dean's Honour List
2011

Queen's University Excellence Scholarship
2010

**Related Work
Experience**

Lecturer, Bio 4436 Evolutionary Behavioural Ecology
The University of Western Ontario
2025

Teaching Assistant
The University of Western Ontario
2015-2024

Publications:

Zanette, L.Y., Hobbs, E.C., **Witterick, L.E.**, MacDougall-Shackleton, S.A., Clinchy, M.
(2019) Predator-induced fear causes PTSD-like changes in the brains and behaviour of
wild animals. Scientific Reports