HIPPOCAMPAL VOLUME AND CELL PROLIFERATION IN A MIGRATORY SONGBIRD: DEVELOPMENTAL CHANGES AND EFFECTS OF A NEUROTOXIC INSECTICIDE

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Table of Contents

Abstract

The hippocampus is a brain region important for spatial cognition. Birds are good models to study the hippocampus due to the range of behaviours that rely on spatial cognition, including migration, food storing, and nest site selection. Yet, the brain is susceptible to negative effects from developmental exposure to environmental contaminants, including insecticides. However, there are limited data that examine the developmental processes in the hippocampus of migratory birds, and how exposure to insecticides may alter normal developmental processes in the hippocampus. Thus, in chapter 2 of this thesis, I evaluated developmental changes in hippocampal volume and hippocampal cell proliferation during post-hatching nestling development in a migratory population of European starlings (*Sturnus vulgaris*). In addition, I examined whether exposure to a neurotoxic insecticide, chlorpyrifos (CPF), disrupts developmental changes in body mass growth and the same measures in the hippocampus. During May and June 2020, starling nest boxes were monitored daily, and on post-hatch day (PHD) 1-3, half the nestlings received an acute sublethal exposure to CPF (6 mg/kg-body weight (bw)), and the remaining nestlings received the same volume of control oil. Blood samples were collected ~16 hours later (PHD 2-4). Then, a subset of nestlings in each treatment group were euthanized in early (PHD 5-7), middle (PHD 11-13) and late (day PHD 19-20) age classes during the nestling period for brain collection, although middle age class brain samples were not processed as part of this thesis. Brains were then sectioned and stained for 4′,6-diamidino-2-phenylindole (DAPI) and 5-ethynyl-2'-deoxyuridine (EdU) to quantify hippocampal volume and hippocampal cell proliferation, respectively. We confirmed the efficacy of CPF exposure with ChE activity, which was significantly lower in CPF-dosed nestlings relative to control nestlings. Moreover, male CPF-dosed nestlings were more affected than female CPF-dosed nestlings. We did not find

effects of CPF exposure on body mass. Moreover, we did not detect developmental changes in corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume) across age classes; however, we found that corrected hippocampal volume was larger in CPF-dosed nestlings relative to control nestlings in the late age class but not in the early age class. The corrected number of EdU^+ cells (i.e., number of EdU^+ cells corrected for hippocampal volume) decreased across age classes in control nestlings. We did not find any difference in the corrected number of EdU^+ cells between control nestlings and CPF-dosed nestlings in the early or late age classes. However, unlike control nestlings, the corrected number of EdU^+ cells did not decrease across age classes in CPF-dosed nestlings. Together, the volumetric and cell proliferation data suggest there was later, compensatory growth in the hippocampus due to CPF exposure. Overall, these results further our understanding of developmental processes that occur during the nestling period in an altricial migratory species. Furthermore, they provide evidence that a single exposure to an insecticide can disrupt development of the hippocampus, a brain region essential for migration, thus potentially having long-tern fitness consequences.

Résumé

L'hippocampe est une région cérébrale importante pour la cognition spatiale. Les oiseaux sont de bons modèles pour étudier l'hippocampe en raison de la gamme de comportements qui reposent sur la cognition spatiale, notamment la migration, le stockage de nourriture et la sélection du site de nidification. Pourtant, le cerveau est sensible aux effets négatifs de l'exposition développementale aux contaminants environnementaux, notamment aux insecticides. Cependant, il existe peu de données examinant les processus de développement dans l'hippocampe des oiseaux migrateurs et la manière dont l'exposition aux insecticides peut altérer les processus de développement normaux dans l'hippocampe. Ainsi, dans le chapitre 2 de cette thèse, j'ai évalué les changements développementaux dans le volume de l'hippocampe et la prolifération des cellules de l'hippocampe au cours du développement des nids après l'éclosion dans une population migratrice d'étourneaux sansonnets européens (*Sturnus vulgaris*). De plus, j'ai examiné si l'exposition à un insecticide neurotoxique, le chlorpyrifos (CPF), perturbait les changements développementaux dans la croissance de la masse corporelle et les mêmes mesures dans l'hippocampe. En mai et juin 2020, les nichoirs d'étourneaux ont été surveillés quotidiennement et, les jours 1 à 3 après l'éclosion (PHD), la moitié des oisillons ont reçu une exposition subléthale aiguë au CPF (6 mg/kg de poids corporel (pc)), et les oisillons restants ont reçu le même volume d'huile témoin. Des échantillons de sang ont été prélevés environ 16 heures plus tard (PHD 2-4). Ensuite, un sous-ensemble d'oisillons dans chaque groupe de traitement a été euthanasié dans les classes d'âge précoce (PHD 5-7), moyenne (PHD 11-13) et tardive (jour PHD 19-20) pendant la période de nidification pour la collecte du cerveau, bien que l'âge moyen les échantillons de cerveau de classe n'ont pas été traités dans le cadre de cette thèse. Les cerveaux ont ensuite été sectionnés et colorés pour le 4′, 6-diamidino-2-phénylindole

(DAPI) et la 5-éthynyl-2'-désoxyuridine (EdU) afin de quantifier le volume de l'hippocampe et la prolifération des cellules de l'hippocampe, respectivement. Nous avons confirmé l'efficacité de l'exposition au CPF avec l'activité de la ChE, qui était significativement plus faible chez les oisillons dosés au CPF par rapport aux oisillons témoins. De plus, les oisillons mâles ont été plus affectés que les oisillons femelles. Nous n'avons pas trouvé un effet de l'exposition au CPF sur la masse corporelle. De plus, nous n'avons pas détecté de changements développementaux dans le volume hippocampique corrigé (c'est-à-dire le volume hippocampique corrigé du volume du télencéphale) dans toutes les classes d'âge; cependant, nous avons constaté que le volume corrigé de l'hippocampe était plus grand chez les oisillons traités par CPF que chez les oisillons témoins dans la classe d'âge tardive, mais pas dans la classe d'âge précoce. Le nombre corrigé de cellules EdU^+ (c'est-à-dire le nombre de cellules EdU^+ corrigé en fonction du volume de l'hippocampe) a diminué selon les classes d'âge chez les oisillons témoins. Nous n'avons trouvé aucune différence dans le nombre corrigé de cellules EdU^+ entre les oisillons témoins et les oisillons traités par CPF dans les classes d'âge précoces ou tardives. Cependant, contrairement aux oisillons témoins, le nombre corrigé de cellules EdU^+ n'a pas diminué selon les classes d'âge chez les oisillons traités par CPF. Ensemble, les données volumétriques et de prolifération cellulaire suggèrent qu'il y a eu une croissance compensatoire plus tardive dans l'hippocampe en raison de l'exposition au CPF. Dans l'ensemble, ces résultats approfondissent notre compréhension des processus de développement qui se produisent pendant la période de nidification chez une espèce migratrice nidicole. En outre, ils prouvent qu'une seule exposition à un insecticide peut perturber le développement de l'hippocampe, une région du cerveau essentielle à la migration, pouvant ainsi avoir des conséquences à long terme sur le succès reproducteur.

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Contribution of authors

Chapter 1: General introduction

Sereena Moore wrote this chapter with feedback from Claire Bottini, Margaret Eng, Christy Morrissey and Mélanie Guigueno.

Chapter 2: Chlorpyrifos exposure disrupts developmental changes in hippocampal volume and cell proliferation in migratory European starling (S*turnus vulgaris)* nestlings

Sereena Moore, Margaret Eng, Christy Morrissey and Mélanie Guigueno designed the study. Margaret Eng and Christy Morrissey conducted the field work. Sereena Moore completed lab work. Sereena Moore and Claire Bottini conducted microscopy. Sereena Moore analysed the data with guidance from Claire Bottini, Margaret Eng, Christy Morrissey and Mélanie Guigueno. Sereena Moore wrote the chapter with feedback from Claire Bottini, Margaret Eng, Christy Morrissey and Mélanie Guigueno. All other acknowledgements are included at the end of the chapter.

Chapter 3: General discussion

Sereena Moore wrote this chapter with feedback from Claire Bottini, Margaret Eng, Christy Morrissey and Mélanie Guigueno.

List of figures

horizontal lines indicate the median and whiskers indicate range, with individual jittered datapoints points overlaid. Boxplot colour indicates sex: female (pink), male (blue). 'X' indicates mean ChE activity. Asterisks indicate significant differences between groups (***: $p < 0.001$; *: $p < 0.05$) and NS (i.e. not significant) indicates no significant difference between groups. Note that raw data is plotted in graph; however, ChE activity was square-root transformed for statistical analysis.

Fig. 5: Body mass (g) measured immediately pre-dosing (post-hatch day (PHD) 1-3), on day of blood sampling (16 hours post-dosing, PHD 2-4), early age class (4 days post-dosing, PHD 5-7), middle age class (10 days post-dosing, PHD 11-13) and late age class (18 days post-dosing, PHD 19-20) in nestling European starlings after exposure to either a control or chlorpyrifos (CPF) dose. Boxplots indicate 25th, 50th, and 75th percentiles, horizontal lines indicate the median and whiskers indicate range, with individual jittered datapoints points overlaid. Boxplot colour indicates sex: female (pink), male (blue). X indicates mean body mass. Asterisks indicate significant differences between groups (***: $p < 0.001$; **: $p < 0.01$) and NS (i.e. not significant) indicates no significant difference between groups. Treatment group was not included in the figure because it did not significantly affect body mass.

Fig. 6: Corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume) in nestling European starling brains collected on the early age class (post-hatch day (PHD) 5-7) and late age class (PHD 19-20) European starling nestlings after exposure to either a

Fig. S4: Diagnostic plots used to validate the linear mixed effects model analyzing the effect of the treatment group by age class interaction on the corrected number of EdU^+ cells (i.e. number of EdU^+ cells corrected for hippocampal volume) with a random effect of nest ID. a) Quantile-quantile plot showing distribution of model residuals used to check for residual normality (*Shapiro-Wilk test p* = 0.0017). b) Quantile-quantile plot showing distribution of random effect (nest ID) residuals used to check for residual normality. c) Plot of residuals versus fitted values used to check for homogeneity.

List of tables

CHAPTER 1: GENERAL INTRODUCTION

Developmental exposure to environmental contaminants can have various adverse effects on wildlife, including effects on brain and behaviour (e.g. Eng et al., 2018; Iwaniuk et al., 2006; Flahr et al., 2015). However, little is known about how exposure to environmental contaminants affects developmental changes of large- and fine-scale measures in the brain of wildlife. Better understanding of how contaminants affect the brain could help identify mechanistic links between contaminant exposure and effects. Thus, in this thesis I 1) examine developmental changes in the hippocampus, with a focus on a large-scale (volume) and a fine-scale (cell proliferation) measure, during post-hatching nestling development in a migratory population of European starlings (*Sturnus vulgaris*) ("starlings" hereafter), and 2) examine how exposure to a neurotoxic insecticide, chlorpyrifos, may disrupt developmental changes in body mass and the hippocampus during post-hatching nestling development.

Cell proliferation, neurons, and glia

Cell proliferation, the process by which new cells are formed within the body, is an important process that occurs during brain development. In the avian brain, new cells are produced in the ventricular zone (reviewed in Doetsch & Scharff, 2002). Newly produced cells go through the cell cycle which consists of four steps in the order of the growth 1 (G1), synthesis (S), growth 2 (G2) and mitosis (M) phases (Fig. 1) (Alberts et al., 2002). The S phase includes deoxyribonucleic acid (DNA) replication (Fig. 1) (reviewed in Laskey et al., 1989). The M phase includes the process of mitosis (Alberts et al., 2002). The G1 and G2 phases are growth phases and are considered the preparatory phases for their respective subsequent phases (Fig. 1) (Alberts et al., 2002). Completion of all four phases is necessary for cells to develop.

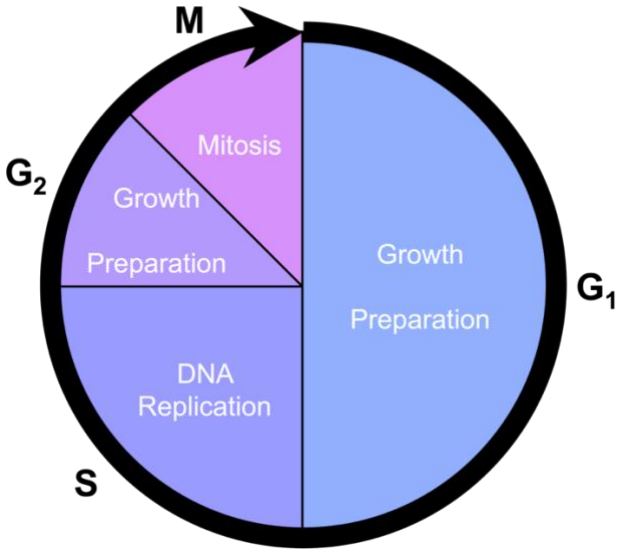

Fig. 1: Schematic diagram depicting four phases of the cell cycle: growth 1 (G1) phase, synthesis (S) phase, growth 2 (G2) phase and mitosis (M) phase. Diagram inspired by Alberts et al., 2002; Shafer, 1998.

Cells that survive the entire cell cycle can differentiate into more mature brain cell types, including neurons and glia. In general, neurons play an important role in transmitting and processing information (Koch & Segev, 2000) and neurogenesis, the production of new neurons, is linked to important cognitive processes, such as learning (Vukovic et al., 2013), memory (LaDage et al., 2010) and forgetting (Akers et al., 2014). Glia have many other functions including supporting neuronal migration, coordinating neuronal differentiation (reviewed in Allen & Lyons, 2018), development (reviewed in Lenz & Nelson, 2018) and memory formation (Suzuki et al., 2011). Moreover, there are many types of glia, including, microglia, oligodendrocyte progenitor cells, radial glia, astrocytes, and oligodendrocytes, and each can play an important role in glial functions (reviewed in Allen & Lyons, 2018). Overall, neurons and glia are fundamental to the function of the brain.

Researchers have developed numerous methods using exogenous and endogenous markers to study fine-scale measures in the brain, including cell proliferation, and neuronal and glial development. The most used exogenous markers of cell proliferation are thymidine analogs, which get incorporated into replicating DNA of proliferating cells during the cell cycle (reviewed in Cavanagh et al., 2011). Notable thymidine analogs include [^{3}H]-thymidine, 5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2′-deoxyuridine (EdU) (Fig. 2) (reviewed in Cavanagh et al., 2011). [^{3}H]-thymidine uses a radiolabelled thymidine which gets incorporated into cells and requires autoradiography to visualize labeled cells (Altman & Das, 1965; reviewed in Cavanagh et al., 2011; Taylor et al., 1957). More recently discovered thymidine analogs include BrdU, where BrdU-incorporated cells are labelled using immunohistochemical staining with a BrdU antibody (Gratzner, 1982) and EdU, where EdU-incorporated cells are labelled with a click-IT reaction which utilizes a fluorescent azide, a reducing compound and copper (II) sulfate (Buck et al., 2008). Alternatively, endogenous markers are those that are naturally expressed within cells, and immunolabeling with antibodies specific to endogenous markers can be used to examine different stages of cell proliferation, differentiation and maturation (Fig. 2) (reviewed in von Bohlen und Halbach, 2011). Endogenous markers for stages in cell proliferation include proliferating cell nuclear antigen (PCNA) and antigen kiel 67 (Ki-67) and markers expressed in progenitor cells include nestin, glial fibrillary acidic protein (GFAP) (Fig. 2) (reviewed in von Bohlen Und Halbach, 2007; reviewed in von Bohlen und Halbach, 2011). Similarly, markers for the neuronal lineage, and those that are expressed in immature and migrating neurons, include the polysialylated neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX) (reviewed in von Bohlen Und Halbach, 2007; reviewed in von Bohlen und Halbach, 2011; Gleeson et al., 1999), and markers used for cells in the glial lineage include

vimentin (Fig. 2) (reviewed in von Bohlen und Halbach, 2011). In mature neurons, the marker neuronal nuclei (NeuN) and calbindin can be used whereas in mature glial cells, markers such as GFAP and s100beta can be used (Fig. 2) (reviewed in von Bohlen und Halbach, 2011). Expression of these markers can often overlap (reviewed in von Bohlen Und Halbach, 2007; reviewed in von Bohlen und Halbach, 2011). However, these exogenous and endogenous markers are useful for studying brain cells.

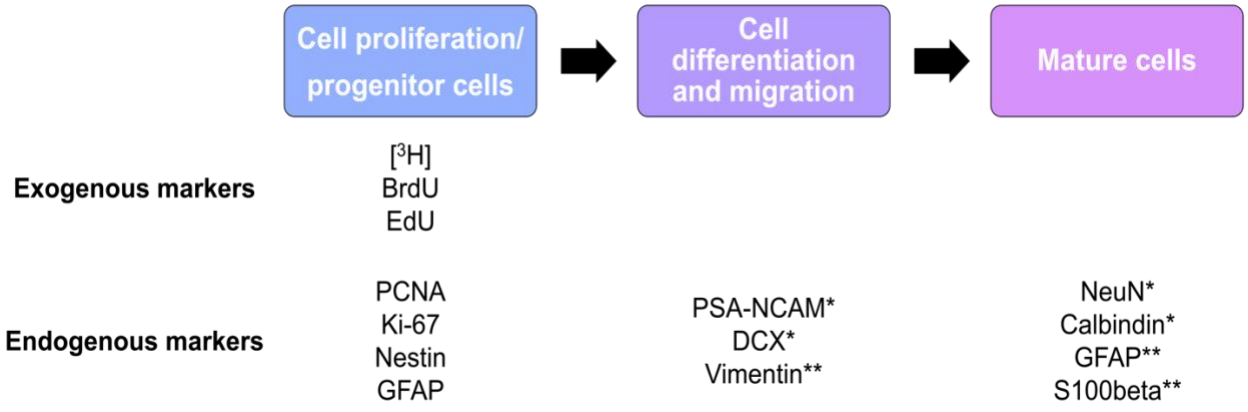

Fig. 2: Common exogenous and endogenous markers that can be used to examine cell proliferation, neurons and glia. One asterisk indicates markers that target neurons, two asterisks indicates markers that target glia, all other markers target all cells. Abbreviations: [^{3}H] = [^{3}H]-thymidine; BrdU = 5-bromo-2'-deoxyuridine; EdU = 5-ethynyl-2′-deoxyuridine; PCNA = proliferating cell nuclear antigen; Ki-67 = antigen kiel 67; GFAP = glial fibrillary acidic protein; PSA-NCAM = polysialylated neural cell adhesion molecule; DCX = doublecortin, NeuN = neuronal nuclei. Data obtained from Balthazart & Ball, 2014; Kempermann et al., 2004; von Bohlen und Halbach, 2011.

The literature provides evidence that fine-scale measures can be influenced by numerous external factors, including but not limited to environmental enrichment, exercise, diet, stress, and contaminants. For example, female mice raised in enriched environment for 40 days starting at post-natal day (PND) 21 had a significantly higher number of proliferating cells that survived in the hippocampal granule cell layer four weeks after BrdU injection compared to controls (Kempermann et al., 1997). Experience with flight exercise in adult male starlings increased the number of DCX^+ spherical cells in the caudal nidopallium (Hall et al., 2014). High-fat diets fed to male rats for four weeks decreased cell proliferation in the granule cell layer (Lindqvist et al., 2006). Moreover, food restriction over 12 weeks post-hatching in female broiler breeder chickens (*Gallus gallus domesticus*) decreased the density of newly produced neurons in the rostral end of the hippocampus (Robertson et al., 2017). Restraint stress and corticosterone injections in adult male rats increased the percentage of new oligodendrocytes but decreased the percentage of new neurons in the dentate gyrus (Chetty et al., 2014). Tobacco smoke exposure for two weeks in adolescent male rats increased the number of newly produced astrocytes but decreased the number of newly produced immature and transitioning neurons in the sub-granular zone/granule cell layer (Bruijnzeel et al., 2011). In sum, there are multiple factors that can positively and negatively regulate brain cells.

Avian hippocampus

The vertebrate brain controls behavioural and cognitive abilities that are significant for life history and fitness. One brain region in particular, the hippocampus, has been adapted for advanced spatial cognitive abilities (reviewed in Sherry et al., 1992). Studies have demonstrated the importance of a functional and intact hippocampus for spatial cognitive measures through

experimental lesions (Sherry & Vaccarino, 1989), and demonstrated that measures such as hippocampal volume (Krebs et al., 1989), hippocampal neurogenesis (LaDage et al., 2010), and hippocampal cell proliferation (Drapeau et al., 2003) are associated with spatial cognitive measures. Overall, the hippocampus has been demonstrated to be an important brain region for spatial cognition in vertebrates.

Birds provide a unique opportunity to study the hippocampus as they exhibit a range of behaviours that rely on spatial cognition, including food hoarding, brood parasitism, and migration. Food hoarding birds hoard food in numerous locations and remember the locations of their caches (Sherry, 1984). Hippocampal lesions affect food-storers ability to find their caches (Sherry & Vaccarino, 1989) and new neurons are incorporated into the hippocampus, especially during autumn when food-storing is most prevalent (Barnea & Nottebohm, 1994). Brood-parasitic females lay eggs in other hosts' nests and must be able to correctly find and remember the location of host nests (reviewed in Sherry & Guigueno, 2019). Female brood parasites have better spatial memory than males (Guigueno et al., 2014) and have a larger hippocampal size with higher levels of neurogenesis than non-parasitic species (Guigueno et al., 2016; Reboreda et al., 1996). Seasonal migration allows animals to obtain more resources to enhance individual fitness (reviewed in Alerstam et al., 2003). Migrating bird brains utilize different information from the environment to correctly orient and navigate themselves (reviewed in Mouritsen et al., 2016). Birds have shown fidelity at stopover sites (Cantos & Tellería, 1994), breeding sites (Thompson & Hale, 1989) and wintering sites (Blackburn & Cresswell, 2016) which likely also requires advanced spatial memory. Superior long-term memory abilities (Mettke-Hofmann & Gwinner, 2003) and superior spatial memory (Pravosudov et al., 2006) in a migrant relative to a non-migrant were demonstrated with spatial memory tests. In sum, food-storing, brood

parasitism, and migration are three behaviours exhibited in birds that require advanced spatial cognitive abilities.

Many studies have shown that migration is linked to neurological measures in birds. For example, brain size relative to body size of migratory birds was smaller than in non-migratory species (Sol et al., 2005). However, other studies showed that older migratory Garden warblers (*Sylvia borin*) with migration experience had a greater relative hippocampal volume with higher neuron density and more total neurons (Healy et al., 1996) and migratory Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) had a greater relative hippocampal volume compared to non-migratory Nuttall's white-crowned sparrow (*Z. l. nuttalli*) (Pravosudov et al., 2006). Furthermore, hippocampal neuron density was higher in migratory slate-coloured dark-eyed junco (*Junco hyemalis hyemalis*) compared to the non-migratory Carolina dark-eyed junco (*J. h. carolinensis*) (Cristol et al., 2003). Migratory reed warblers (*Acrocephalus scirpaceus*) had higher hippocampal $BrdU^+$ density compared to the non-migratory Clamorous warbler (*A. stentoreus*) in all seasons measured (i.e. autumn, spring and summer) (Barkan et al., 2014). Moreover, hippocampal DCX^+ density and total hippocampal DCX^+ cells were higher in adult migratory Gambel's white-crowned sparrows compared to adult non-migratory Nuttall's white-crowned sparrows (LaDage et al., 2011). Semipalmated sandpipers (*Calidris pusilla*) had a larger hippocampal volume, and higher number and density of hippocampal DCX^+ labelled cells when measured during wintering relative to those that were measured during migration (Magalhães et al., 2017) and factors experienced during migration influenced certain measures of astrocyte morphology in the semipalmated sandpiper and semipalmated plover *(Charadrius semipalmatus*) (Henrique et al., 2021; Mendes de Lima et al., 2019). In addition, the number of hippocampal parvalbumin inter-neurons, a calcium-binding protein, was lower in the non-migratory period

relative to before migration in the spotted sandpiper (*Actitis macularius)* (Guerreiro et al., 2022). Furthermore, the distance traveled during migration may play a role in hippocampal plasticity, as one study found that hippocampal neuronal recruitment tended to be higher in reed warblers that migrated further distances (Barkan et al., 2016). Overall, these studies demonstrated that numerous neurological measures in the hippocampus are associated with the bird migratory condition.

Avian development

Developmental processes can vary between and within taxa, including in birds. The developmental mode (classification system explaining how developed an individual is at hatching) ranges on a spectrum from super-precocial to altricial (Starck, 1993). Developmental modes differ depending on phylogeny, and each mode displays variation in developmental, physical, behavioural and physiological features, pre- and post-hatching (Ducatez & Field, 2021; Starck, 1993). Precocial species are relatively mature at hatching and require little to no parental care (Starck & Ricklefs, 1998). Common physical characteristics of precocial hatchlings is hatching with open eyes, and hatching with some feathers (Starck & Ricklefs, 1998). Moreover, brain development and production of new brain cells in precocial species occurs primarily in the pre-hatching period; however, the rate at which it occurs varies depending on species (Bennett & Harvey, 1985; Charvet & Striedter, 2010, reviewed in 2011). Precocial brains do not change substantially post-hatching (Bennett & Harvey, 1985; Starck, 1993). In contrast, altricial species are relatively immature at hatching and require more extensive parental care (Starck & Ricklefs, 1998). Common physical characteristics of altricial hatchlings include hatching with closed eyes and hatching without feathers (Starck & Ricklefs, 1998). However, post-hatching growth in

altricial species is quick (Starck, 1993). Brain development begins during egg incubation (Bennett & Harvey, 1985; Murray et al., 2013); however, increases in brain volume and cell production occurs primarily during the post-hatching period (Bennett & Harvey, 1985; reviewed in Charvet & Striedter, 2011; Starck, 1993). Moreover, at hatching, altricial species brains can only function for primitive physiological processes post-hatching (Starck, 1993). Thus, the characteristics exhibited by precocial and altricial species during development are relatively different.

The development of brain regions in birds is largely asynchronous. There are limited data on hippocampal development in birds; however, hippocampal PCNA cell proliferation and DCX neurogenesis density decreased in certain regions of male Japanese quail (*Cortunix japonica*) brains (Nkomozepi et al., 2018) and DCX distribution and intensity also decreased in certain regions of domestic chicks brains (Mezey et al., 2012) after hatching. In homing pigeons (*Columba livia domestica*), the proportion of DCX^+ cells decreased over a longer time scale with age (Meskenaite et al., 2016). In chicken embryos, the majority of neurons in the hippocampus developed from the hippocampal neurogenic zone; however, less proliferating cells form within the hippocampal neurogenic zone compared to the lateral neurogenic zone (Gupta et al., 2012). Another commonly studied set of interconnected brain nuclei (i.e. the song control nuclei) in songbirds have different patterns of development. This has been well-studied in zebra finches (*Taeniopygia guttata*), who are close-ended learners, meaning their song is learned and develops over a specific period in development and does not change later in life (reviewed in Brenowitz et al., 1997). Two pathways involved in song include the motor pathway, implicated in song learning and song production, and the anterior forebrain pathway, implicated in song learning and song identification (reviewed in Brenowitz et al., 1997). The high vocal center (HVC) and

robust nucleus of the arcopallium (RA) are part of the motor pathway (reviewed in Brenowitz et al., 1997), and the HVC volume increases before the RA in the zebra finch (Bottjer et al., 1985). Moreover, increases in volume of the HVC and RA post-hatch is due to an increase in neuron numbers, or a decrease in neuron density with an increase in the size of neurons, respectively (Bottjer et al., 1986). Area X and magnocellular nucleus of the anterior nidopallium (MAN) are two song control nuclei part of the anterior forebrain pathway (reviewed in Brenowitz et al., 1997), and both change in size after the HVC increases in size in zebra finch (Bottjer et al., 1985). These developmental processes are fundamental to forming the specialized structures and functions of different brain regions.

Effects of insecticide exposure on the brain

Since the start of the industrial revolution, anthropogenic contaminants have become a widespread concern. Many contaminants may be unintentionally released, but some, such as insecticides, are intentionally applied to crops to target insect pests, which may result in non-target organism exposure. Many insecticides are neurotoxic and have numerous undesirable effects when non-target organisms are exposed. For example, mass mortalities have been reported in numerous birds that fed on organophosphate (OP) or carbamate treated seeds, granules or contaminated prey (Flickinger et al., 1984; reviewed in Mineau, 2018). One example of an OP neurotoxic insecticide of concern is chlorpyrifos (CPF), which was first introduced in 1966 by Dow Chemical Company (Dow Inc.®) and was a widely used high production volume chemical (reviewed in Hites, 2021). Although CPF has a short atmospheric lifetime and degrades quickly (reviewed in Hites, 2021), it is still of concern. It was designed as an insecticide in agricultural and residential use; however, toxic developmental effects of CPF were later

discovered resulting in the ban of residential uses in 2000 in the US (reviewed in Hites, 2021). Later, CPF became banned in different areas including the European Union, and finally, in 2020, Dow Chemical Company announced it would voluntarily terminate the production of CPF (reviewed in Hites, 2021). In Canada, CPF sales were banned in December 2022 and currently, the product is being phased out (Health Canada, 2021). Thus, although it is highly regulated and even banned in certain jurisdictions, CPF is still a major concern today.

CPF has numerous deleterious effects on non-target organisms. One major characteristic of all OPs, including CPF, is that they are neurotoxic due to their ability to restrict cholinesterase (ChE) enzymes, particularly acetylcholinesterase (AChE) (Mileson et al., 1998). In the case of CPF, it is first converted to a metabolite, CPF-oxon, which phosphorylates the serine hydroxyl group on AChE, rendering AChE inhibited (Testai et al., 2010). Inhibition of AChE causes a build-up of ACh at nicotinic and muscarinic receptors within the nervous system, leading to paralysis and death (Testai et al., 2010). In addition, non-target organisms exposed to sub-lethal concentrations can experience detrimental health effects on behaviour, physiology and at the cellular level. For example, prenatal CPF exposure resulted in a lower intelligence quotient (IQ) in young children (Rauh et al., 2011). CPF exposure altered migratory orientation in migratory Gambels white-crowned sparrow (Eng et al., 2017), and slower flight in homing pigeons (Moye & Pritsos, 2010). Prenatal exposure also caused a sex-specific effect that targeted female rats' reference and working memory in a spatial radial arm maze test later in life (Levin et al., 2002). In broiler chicks, CPF exposure caused lower body mass and a reduction in food consumption at certain time points of the experiment (Ahmad et al., 2015) and hypoactivity (Al-Badrany & Mohammad, 2007). Moreover, postnatal CPF exposure affected the brain on a cellular level, including changes in DNA synthesis and protein synthesis in the forebrain, brainstem and

cerebellum of rats (Whitney et al., 1995). Similarly, post-natal CPF exposure caused changes in DNA and protein amounts in the forebrain, brainstem and cerebellum (Campbell et al., 1997), affected the number of glia and neurons in the hippocampus (Roy et al., 2005), and affected glial cells in numerous regions of the brain (Garcia et al., 2002) of rats. Maternal CPF exposure was shown to affect hippocampal neurogenesis in offspring mice (Wang et al., 2013) and hippocampal cell proliferation in offspring rats (Ohishi et al., 2013); however, these effects were undetected at a later age that was measured. In sum, CPF exposure is a major concern as it has numerous effects on non-target organisms.

Study species

Starlings are an altricial passerine species distributed in different areas of the world (reviewed in Linz et al., 2007). In the beginning of the 1890s, the starling was introduced into New York (reviewed in Linz et al., 2007). Since then, starlings have become widespread throughout North America primarily due to general movements between locations and migration (Kessel, 1953). Starlings in North America can be both resident and migratory (Kessel, 1953). In the prairies, starlings are known to primarily be migratory; whereas in southern Quebec, there are relatively higher abundances of starlings during the wintering period (Fink et al., 2022). Moreover, migratory direction differs between populations. Starling populations that reside in geographical locations that are east of the Appalachian Mountains generally migrate using a northeast-southwest or north-south direction (Kessel, 1953). However, populations that inhabit locations west of the Appalachian mountains generally migrate using a northeast-southwest direction (Kessel, 1953); although there may be variation in certain populations (Flahr et al., 2015). Migratory populations of starlings migrate to their wintering grounds between September

to November and migrate to their breeding grounds between February and March, (Kessel, 1953).

The starling breeding season occurs between March to June (Kessel, 1957). Starlings generally have fidelity for their nestling sites; however, they are also highly flexible and can nest in any structure and around other individuals (Kessel, 1957). Females lay one egg a day and clutch sizes generally range from two to eight eggs in North America with an average of approximately five eggs/clutch; however, clutches of between one to 10 eggs have been documented (Kessel, 1957). Incubation of eggs begins when the last egg or second last egg is laid and is performed by both parents; however, only females incubate the eggs at night (Kessel, 1957). Starlings also may perform conspecific brood parasitism, where a female lays her eggs in the nest of another conspecific (Evans, 1988). At around post-hatch day (PHD) 21, nestlings fledge the nest and adult females may lay a second brood approximately 40-50 days after the first brood (Kessel, 1957). Outside the nest, starlings primarily feed on insect prey but can also eat grains, seeds, and fruits (Cabe, 2020). They also frequently forage as groups (Williamson & Gray, 1975).

Starlings are an optimal model to study the issues discussed previously. Their migratory behaviour has been previously studied (Burtt & Giltz, 1977; Kessel, 1953), and the presence of both migratory and non-migratory populations make them an ideal species to further elucidate the relation between migratory behaviour and the hippocampus. Additionally, starlings have been used in numerous ecotoxicological studies and are suggested to be a good model for assessing effects of contaminants in terrestrial passerines (Arenal et al., 2004; Chen et al., 2013; Eens et al., 2013; Eng et al., 2014; Flahr et al., 2015; Wolfe & Kendall, 2009; Zahara et al., 2015). Starlings in Europe have been noted to be declining due to agricultural practices (Donald

et al., 2006), thus, examining the effects of an agricultural insecticide, like CPF, is important and relevant as exposure may negatively affect fitness-related behaviours and cognition. Thus, starlings make a good model to examine developmental processes during the nestling period and the effects of agricultural insecticides on the brain.

Thesis overview

This thesis is divided into the following two objectives: 1) to determine whether there are developmental changes in a large-scale (volume) and a fine-scale (cell proliferation) measure in the hippocampus during post-hatching nestling development and 2) to determine if early acute sublethal CPF exposure disrupts developmental changes in body mass and the hippocampus during post-hatching nestling development. In chapter 2 of the thesis, I examine both objectives. I used a free-living migratory population of starling nestlings to examine these measures in multiple age classes over post-hatching nestling development. In chapter 3 of this thesis, I review the results of the thesis, the broader implications, the limitations, and discuss future directions that should be taken to examine these objectives further. The results of this thesis are important in improving our understanding of the development of brain regions that are significant for fitness in altricial birds. Furthermore, by combining field and laboratory approaches, this thesis makes clear linkages between exposure to insecticides under natural conditions to effects on the brain during a critical period of development.

CHAPTER 2: CHLORPYRIFOS EXPOSURE DISRUPTS DEVELOPMENTAL CHANGES IN HIPPOCAMPAL VOLUME AND CELL PROLIFERATION IN MIGRATORY EUROPEAN STARLING (S*TURNUS VULGARIS)* NESTLINGS

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Highlights

- Efficacy of chlorpyrifos (CPF) exposure was confirmed by a decrease in ChE activity in blood samples taken the day after exposure, with males being more affected than females.
- CPF exposure had no effect on body mass in any age classes measured.
- Among the oldest nestlings (PHD 19-20), corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume) was larger in CPF-dosed nestlings compared to the control nestlings; however, we did not find any developmental changes in corrected hippocampal volume across age classes.
- The corrected number of 5-ethynyl-2'-deoxyuridine $(EdU)^+$ cells (i.e. number of EdU^+ cells corrected for hippocampal volume), a measure of cell proliferation, decreased across age classes in control nestlings, but not in CPF-dosed nestlings.
- Overall, CPF affected normal developmental changes in hippocampal volume but did not affect hippocampal cell proliferation.

Abstract

Developmental exposure to neurotoxic insecticides, such as the organophosphate chlorpyrifos (CPF), can cause deleterious effects on the behaviour and brain of non-target organisms. Migratory birds may have increased risk of exposure due to their extensive space use across seasons. However, there is limited understanding of how acute sublethal exposure to neurotoxic insecticides affects neural mechanisms that underlie fitness-related behaviour in migratory birds, especially during development. One behaviour essential for migration is spatial cognition, which is controlled primarily by the hippocampus, a brain region located on the dorsal surface of the bird brain. In this study, we aimed to examine changes in the hippocampus over

post-hatching nestling development and the effects of acute sublethal exposure to CPF. We exposed a migratory population of European starling (*Sturnus vulgaris)* nestlings to CPF (6 mg/kg-body weight (bw)) soon after hatching (post-hatch day (PHD) 1-3). We collected blood samples to measure cholinesterase (ChE) activity approximately 16 hours after dosing (PHD 2-4) and collected brain samples from nestlings at the early (PHD 5-7), middle (PHD 11-13) and late (PHD 19-20) age classes during the nestling period to examine effects on hippocampal volume and proliferating cell density using 4′,6-diamidino-2-phenylin-dole (DAPI) and 5-ethynyl-2'-deoxyuridine (EdU), respectively. Body mass was also measured at all sampling points. We confirmed CPF exposure through a decrease in ChE activity, with males ChE activity being lower than females after exposure. CPF exposure did not affect developmental changes in body mass. Moreover, we did not detect developmental changes in corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume); however, corrected hippocampal volume was larger in late age class CPF-dosed nestlings relative to control nestlings. The corrected number of EdU^+ cells (i.e. number of EdU^+ cells corrected for hippocampal volume) decreased across age classes in control nestlings; however, it did not decrease across age classes in CPF-dosed nestlings. These results demonstrate that even a single dose to a neurotoxic insecticide can disturb normal developmental processes in the brain. These effects can potentially have negative consequences on migratory birds' cognitive performance and survival.

Keywords: Cell proliferation, chlorpyrifos, development, hippocampus, migration, European startling

1. Introduction

Organophosphate (OP) insecticides are a class of insecticides widely used in the agricultural industry. After exposure, OPs inhibit cholinesterase's (ChE), particularly acetylcholinesterase (AChE), an enzyme responsible for acetylcholine (ACh) neurotransmitter breakdown (Mileson et al., 1998). AChE inhibition results in cholinergic overstimulation at nicotinic and muscarinic receptors within the nervous system due to a build-up of ACh, thus affecting normal cell signaling in the brain (Mileson et al., 1998). In addition to ChE inhibition, OPs like chlorpyrifos (CPF) can have effects unrelated to ChE inhibition (Slotkin & Seidler, 2012). The effects that OP insecticides like CPF have on non-target organisms are thus of concern.

The effects of CPF exposure have been well documented in mammals. Studies on rodent models have demonstrated that developing individuals exposed to CPF pre-natally and post-natally are highly susceptible to effects on brain cells. For example, post-natal CPF exposure inhibited deoxyribonucleic acid (DNA) and protein synthesis (Whitney et al 1995) and altered DNA and protein concentrations (Campbell et al., 1997) within different brain regions in rats. Pre- and post-natal CPF exposure affected neurogenesis (Wang et al., 2013), hippocampal cell proliferation (Ohishi et al., 2013), and glial cells (Garcia et al., 2002) in rodents. Neurogenesis, the production of new neurons, is linked to learning (Vukovic et al., 2013), memory (Deng et al., 2009) and forgetting (Akers et al., 2014). Cell proliferation, the production of new brain cells including neurons and glial cells, are also linked to learning (Gould et al., 1999) and memory (Drapeau et al., 2003). Glial cells have many other roles in the brain, such as homeostasis (reviewed in Allen & Lyons, 2018), development (reviewed in Lenz & Nelson, 2018) and

memory (Suzuki et al., 2011). Thus, any effects that CPF exposure has on brain cells can impact numerous important functions in the brain.

Although the cellular effects of CPF exposure have been well studied in mammalian brains, it is still unknown how CPF can affect brain regions that control ecologically-relevant behaviour in birds. The hippocampus is a brain region that is essential for spatial cognition, a cognitive trait linked to several fitness-related behaviours in birds, including migration, food-storing behaviour, and nest site selection. In birds that rely on spatial cognition, hippocampal volume is larger (Krebs et al., 1989; Reboreda et al., 1996) and hippocampal neurogenesis (LaDage et al., 2011) and hippocampal cell proliferation (Patel et al., 1997) are higher. In particular, migratory birds have a larger hippocampal volume (Pravosudov et al., 2006), a higher neuron density (Cristol et al., 2003) and higher levels of neurogenesis (LaDage et al., 2011) relative to non-migrants. Furthermore, migration has been shown to affect the number and morphological characteristics of hippocampal astrocytes, a type of glial cell (Carvalho-Paulo et al., 2018). Thus, the importance of the hippocampus in migrants makes them a suitable model to elucidate the effects of CPF on the hippocampus.

Migratory birds may be at a high risk of CPF exposure due to their use of different environments across seasons. Even short-term exposure to CPF could have consequences for behaviour and spatial cognition, directly or indirectly. Exposure to a low and high CPF dose during migration in white-crowned sparrows (*Zonotrichia leucophrys)* caused a lack of directional migratory orientation (Eng et al., 2017). Similarly, exposure to a sub-lethal dose of CPF resulted in longer flying time in homing pigeons (*Columbia livia domestica*) (Moye & Pritsos, 2010). Furthermore, CPF had effects on body mass, food consumption (Ahmad et al., 2015; Richards et al., 2000) and hypoactivity (Al-Badrany & Mohammad, 2007) in broiler

chicks (*Gallus gallus domesticus*), which could potentially indirectly affect long-term spatial cognition and the hippocampus (Pravosudov et al., 2005). Any direct or indirect impairments on migratory orientation, flight speed and spatial cognition by CPF could have negative consequences on the ability of migratory birds to successfully complete migration.

There are limited studies on how CPF affects neural mechanisms in the brain of birds and the majority have focused on poultry. Researchers showed that CPF exposure inhibited brain ChE activity (Al-Badrany & Mohammad, 2007) and caused necrosis in the brain (Ahmad et al., 2015) of broiler chicks. Furthermore, CPF induced changes in gene expression of numerous genes, including those important for neurogenesis in broiler chickens and *Fayoumi* chickens (Pinkas et al., 2015; Rimawi et al., 2023). However, no study has examined the effects of CPF exposure on the hippocampus of migratory birds.

By looking at the effects of CPF exposure on the brain of migratory birds, we can examine the underlying mechanisms through which CPF can affect behaviour. The overall goal of this study was to examine how early acute exposure to CPF can affect the hippocampus, and more specifically, hippocampal volume and proliferating cells within the hippocampus, during nestling development in a migratory songbird, the European starling (*Sturnus vulgaris)* ("starlings hereafter). We exposed nestlings to an acute sublethal dose of CPF the day after hatching (post-hatch day (PHD) 1-3) in nest boxes, then took blood samples the day after (PHD 2-4) to confirm dosing with ChE activity. We measured body mass pre-dosing (PHD 1-3), day of blood sampling (PHD 2-4), and in early (PHD 5-7), middle (PHD 11-13) and late (PHD 19-20) age classes during the nestling period until fledging. We also collected brain samples from the early, middle and late age class nestlings. We used 4′,6-diamidino-2-phenylindole (DAPI) to measure brain region volumes. We also used an exogenous marker of cell proliferation, 5-

ethynyl-2’-deoxyuridine (EdU), to label proliferating cells in the process of DNA replication produced immediately prior to brain collection (Buck et al., 2008) and unbiased stereology to count the number of proliferating cells. Because CPF has been shown to decrease DNA synthesis (Whitney et al., 1995) and EdU is incorporated into cells during DNA replication, we predicted that CPF would negatively affect hippocampal cell proliferation throughout the entire post-CPF exposure nestling period, resulting in lower hippocampal volume and cell proliferation.

2. Methods

2.1. Study species and study site

Between May and June 2020, we worked with a population of migratory starling nestlings on the Goodale Farm at the University of Saskatchewan (52°03'21.4"N 106°30'21.4"W) near Saskatoon, Saskatchewan, Canada. We chose to work with starlings for several reasons. First, because they are migrants in our study region (Fink et al., 2022), examining the effects of CPF on the hippocampus, a brain region highly important for migration, was especially relevant. Second, they nest in nest boxes at the study site, providing the opportunity to examine developmental processes during the nestling period. Finally, starlings are found in agricultural landscapes (Jobin et al., 2001), thus, examining the effects of an agricultural insecticide, like CPF, is environmentally relevant.

The nestlings used in this study came from 22 nests distributed throughout the study site. Nestlings from the first and second clutch of two nests were used and nestlings from only the second clutch of one nest were used. Nestlings from the first clutch for the remaining nests were used. Repeated measures were taken for each nestling; however, sample sizes decreased throughout the study timeline as brains were collected (Fig. 1). Dosing was performed and field

measurements (i.e. body mass) and samples (i.e. blood samples and brain samples) were collected throughout the nestling period, beginning at post-hatch day (PHD) 1 and ending just before fledging (PHD 20) (Fig. 1). Once birds were dosed (PHD 1-3), all measurements and samples were collected at a standardized time from (i.e. 16 hours, 4 days, 10 days, 18 days). All work was approved by the University of Saskatchewan Animal Research Ethics Board (Animal Use Permit # 20110043).

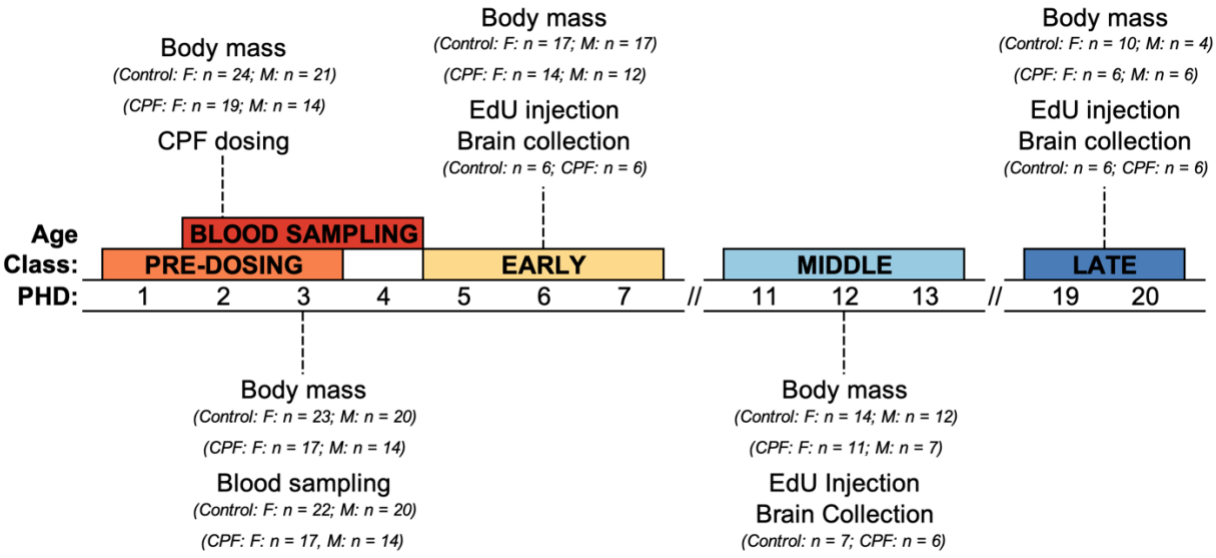

Fig. 1: Timeline of field work where vehicle control and chlorpyrifos (CPF) dosing was performed, and where all measurements (i.e. body mass) or samples (i.e. blood and brain samples) were collected throughout the nestling period. Colours indicate different age classes, and width of coloured bars indicate range of days where each step was performed, and each measurement or sample type was collected. After dosing was completed (PHD 1-3), all measurements and samples were collected at a standardized time from dosing (i.e. 16 hours, 4 days, 10 days, 18 days). Sample sizes for each measurement or sample type are included below

their corresponding measurement or sample type. Abbreviations: PHD = post-hatch day; F = female; M = male.

2.2. CPF dosing

We monitored nests for egg laying and checked nests daily during late incubation to identify hatch date (hatch day = day 0). We used nestlings from nests that had either five or six nestlings. Between 4pm and 6pm on PHD 1-3, nestlings were dosed via oral gavage using a curved 20-gauge, 3.8 cm stainless steel crop feeding tube. Either two out of five or three out of six of all the nestlings in each nest were dosed with CPF at a target dose of 6 mg/kg-body weight (bw) with a dosing volume of 5µl/g-bw and nominal dosing solution concentration of 1.2 µg/µl. CPF dosing solutions were made by dissolving analytical grade CPF (CAS 2921-88-2, Sigma Aldrich, St. Louis, Missouri, USA) in a small volume of acetone, then diluted in the vehicle, organic food-grade sunflower oil (Compliments brand, Sobeys Canada). Solutions were stirred overnight to allow acetone to evaporate and were stored in amber glass bottles in the dark for the duration of the study. The remaining nestlings in each nest were given the equivalent volume of vehicle sunflower oil as a control. Immediately pre-dosing (PHD 1-3; referred to as "pre-dosing" age class; Fig. 1), we took standard morphological measurements, including body mass, wing length, and tarsus length.

The target dose was selected to be in a range that could cause sublethal effects, without causing acute lethality. Reported median lethal doses (LD50) for CPF in starlings have a wide range, from 5 mg/kg-bw (Schafer, 1972) to 75 mg/kg-bw (reviewed in Schafer & Brunton, 1979). A study in nestling starlings dosed with 2.0 mg/kg-bw found 90% survival, which was the same as the control group (Meyers et al., 1992). Because of the uncertainty in starling toxicity

thresholds, we conducted a small range-finding pilot dosing test using an early season nest. Candidate doses were 2, 6 or 9 mg/kg-bw, with n = 2 nestlings per dose. The highest dose that did not result in acutely toxic effects (i.e. 6 mg/kg-bw) was chosen as the target CPF dose for the study.

2.3. ChE activity

We used ChE activity to confirm CPF exposure the day after dosing (Fig. 1). Nestlings were blood sampled between 8:45 am to 11:15 am ~16-17 hours post-dosing (PHD 2-4; referred to as "blood sampling" age class; Fig. 1) from the jugular vein with heparinized 27G needle and syringe. ChE blood samples were taken in the morning to account for diurnal changes in blood ChE levels (Thompson et al., 1988). A maximum of 10% blood volume (1% body mass) was collected per nestling. Blood was stored on ice packs and centrifuged within two hours to separate plasma from red blood cells. Blood samples were stored at -80°C until analysis. We repeated standard measurements the same day (i.e. body mass, wing length, tarsus length).

ChE activity (mU/mL) in plasma was measured using a colorimetric ChE assay kit (ab138871, Abcam; Cambridge, United Kingdom) following the manufacturer's instructions. We used unknown plasma that was diluted 100x in the assay buffer and ran it in duplicates. Additionally, we used in-house captive starling plasma pools as reference materials in each 96-well plate to assess inter-assay and intra-assay variation. We found inter-assay variation to be 3.5% and intra-assay variation to be 4.6%.

2.4. EdU dosing and brain sample collection

We examined changes in hippocampal cell proliferation during the nestling period after CPF dosing using EdU staining. Between ~8 am to 12 pm, four days post-dosing (PHD 5-7; referred to as "early" age class; Fig. 1), one vehicle control-dosed and CPF-dosed nestling in each nest was given an intramuscular injection into the pectoral muscle with EdU at a dose of 50 mg/kg. A fresh solution of EdU (CAS 61135-33-9, Abcam, ab146186) in phosphate-buffered saline (PBS) was made daily at a concentration of 20mg/mL, and injection volume was 2.5 mL/kg-bw. Nestlings were returned to the nest. Then ~2 hours 40 minutes post-EdU injection, nestlings were collected and brought to the Facility for Applied Avian Research (FAAR) for brain collection. Nestlings were deeply anaesthetized with isofluorane (5% iso @ 0.6 LPM O2), transcardially perfused with cold (4°C) heparinized (10 U/mL) 0.1M PBS (pH = 7.4), followed by cold buffered 4% paraformaldehyde (pH = 8.5). Brains were removed from the skull and post-fixed for 24 hours in 4% paraformaldehyde at 4°C. Brains were cryoprotected in 30% sucrose in PBS (pH = 7.4) at 4°C until they sank to bottom of vial, then flash frozen on powdered dry ice and stored at -80°C. We repeated the standard morphological measurements the same day (i.e. body mass, wing length, tarsus length). Sex was determined during brain dissections by inspecting gonads.

The steps were repeated on 10 days post-dosing (PHD 11-13; referred to as "middle" age class; Fig. 1) and 18 days post-dosing (PHD 19-20; referred to as "late" age class; Fig. 1) with one CPF-dosed and one vehicle control-dosed nestling in the same nests. We did not process brains that were collected in the middle-age class; thus, we are only presenting results from early and late age class nestlings.

2.5. DAPI and EdU staining

We sectioned the frozen nestling brains on a coronal plane at 20 μm using a cryostat (CM1950, Leica Biosystems, Wetzlar, Germany) and collected series that had every 20th (early age class) or every 10th (late age class) section. We collected series at different intervals for early and late age class nestlings for stereology optimization (details below). We completed preliminary staining tests on brains and based on the low EdU^{+} cell density we observed in late age class brains, we decided to collect sections at a smaller interval for optimization. We thaw-mounted sections directly onto Superfrost Plus slides (VWR International, Radnor, Pennsylvania, USA) during sectioning with one drop of ultra-pure type 1 water (Barnstead NanoPure, Thermo Fisher Scientific, Waltham, Massachusetts, USA) under each section to flatten them onto the slide. The slides were dried overnight at room temperature then stored at -80°C until use.

We stained each section for EdU and DAPI at room temperature. DAPI is a fluorescent probe that stains all cells by binding to DNA (Kapuscinski, 1995). Each staining round contained a pseudorandomly selected group of brains from different treatment groups. We used one randomly selected series of slides plus extra adjacent slides to accommodate any missing/damaged sections. To start the staining process, we took slides out of the -80°C to dry overnight. The next day, we used a Super HT PAP pen (#22006, Biotium, Freemont, California, USA) to draw a hydrophobic barrier around the outer edge of the slides. We placed slides in a handmade humidity chamber which consisted of a slide box with a damp paper towel inserted on the bottom for all staining steps. We made all solutions with ultrapure type 1 water (Barnstead NanoPure, Thermo Fisher Scientific). We washed slides with 1X PBS three times for 10 minutes each. Then, we performed the EdU staining protocol with an Alexa Fluor 647 EdU staining kit

(C10340, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, we incubated slides with 3% bovine serum albumin (BSA; A8022, Sigma Aldrich) dissolved in 1X PBS twice for 10 minutes each, and permeabilized slides with 0.3% triton dissolved in 1X PBS for 20 minutes. We incubated slides again in 3% BSA in 1X PBS twice for 10 minutes each. Then, we incubated slides with the EdU reaction cocktail for 30 minutes in the dark. The final reagent in the cocktail was added just prior to incubation. All remaining steps were performed with minimal light. We incubated slides in 3% BSA in 1X PBS two more times for 10 minutes each and then washed them with 1X PBS three times for 10 minutes each. We counterstained slides with DAPI ready-made solution (MBD0015, Sigma Aldrich) diluted 1:200 in 1X PBS for 40 minutes. After DAPI incubation, we washed the sections with 1X PBS three times for 10 minutes and once in ultra-pure type 1 water for 10 minutes. Finally, we cover-slipped slides with Prolong Gold Antifade Mounting Medium (P36934, Thermo Fisher Scientific), dried them overnight at room temperature and then stored them in folders at 4°C until imaging.

2.6. Telencephalon scans and volume measurements

We used DAPI staining for telencephalon volume measurements. We used the 2D slide scanning module in the Stereo Investigator (MBF Bioscience, Williston, Vermont, USA) connected to a Zeiss AxioImager M2 microscope (Zeiss, Oberkochen, Germany) system to take full slide scans of DAPI stained sections. We took scans at 2.5x (25x total magnification) with the DAPI channel on to visualize DAPI stained cells (Fig. 2a). We drew a contour around the perimeter of two slides at a time, whenever possible, ensuring that all sections were within the contour. We used 10% overlap percentage for all scans. We focused on tissue at 20% of sites,

enabled the software to randomly select the sites and skipped all sites that were only the background of the slide. Once the scans were complete, we adjusted exposure as necessary.

Telencephalon volumes were measured in Fiji Is Just ImageJ (Fiji) (Schindelin et al., 2012). We used the scaling provided by the Stereo Investigator for each scan and set that as the scale in Fiji. We traced the border of the telencephalon of every 20th section in one series for all brains. When sections were slightly damaged or missing a part of the tissue, we estimated how much was missing based on the previous and subsequent section. If a section was too damaged to estimate, we skipped that section. To calculate the volume of the telencephalon, we used the frustum formula that calculates the volume between each section measurement, and then summed up the volume of each section to obtain total telencephalon volume (Sherry et al., 1989). We completed all telencephalon tracings blind to treatment group, but not age class, because age was identifiable by section size and number of sections.

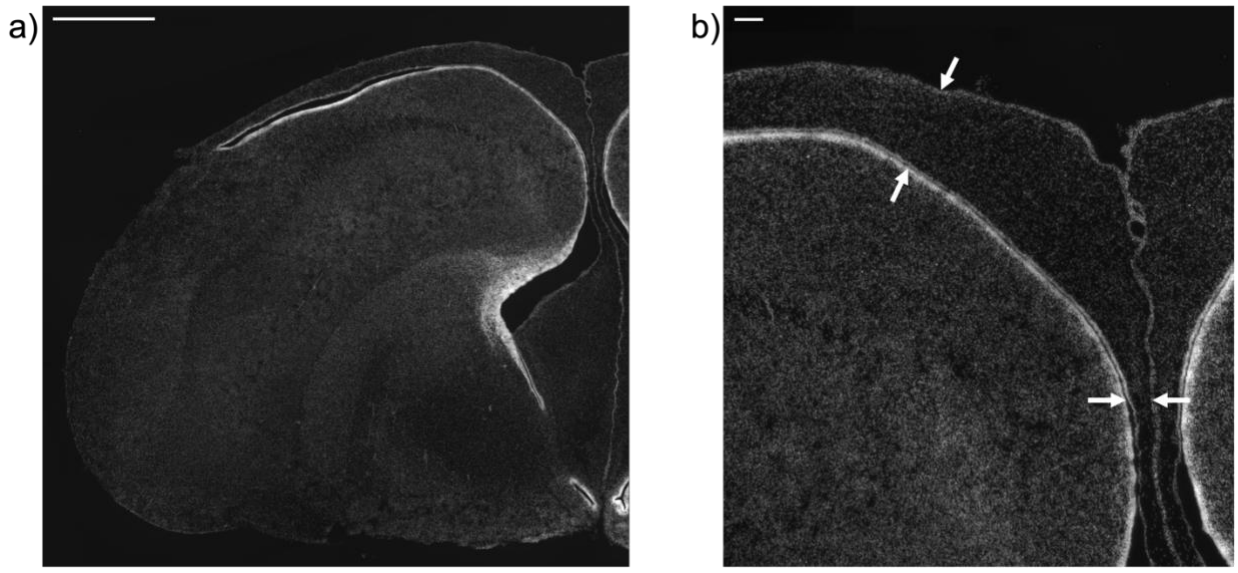

Fig. 2: Example of a) telencephalon and b) hippocampus identified using 4',6-diamidino-2-phenylindole (DAPI) staining. The lateral hippocampal boundary and boundary between the

hippocampus and the septum are identified by arrows. Dorsal boundary is the dorsal edge of the tissue, and ventral boundary is the ventricular zone. Scale bar = 1000 µm (a); 100 µm (b).

2.7. Hippocampal volume

As with the telencephalon, we used DAPI staining for hippocampal volume measurements. First, we outlined the boundary of the hippocampus within Stereo Investigator (MBF Bioscience) with the DAPI channel on to visualize hippocampal boundaries (Fig. 2b). For all sections containing the hippocampus, the lateral boundary was identified by finding the point in which cell density changes (Sherry et al., 1989), and more specifically, by an increase in the number of cells, an appearance of a higher number of small cells, and cells more closely packed. The ventral boundary was the ventricular zone, and the medial boundary was the midline of the brain, and the dorsal boundary was the dorsal edge of the brain. The boundary between the hippocampus and the septum was identified by locating the pinch in tissue and the change in cell density from the hippocampus to the septum. The rostral end of the hippocampus was identified by the first section with the change in cell density on lateral boundary and identifying the shape of the hippocampus, following the carrion crow (*Corvus corone*) atlas (Kersten et al., 2022). The caudal end was identified by finding the final section that had the appearance of the deeply staining V and where the change in cell density on the lateral boundary was still visible. All boundary identifications were done primarily at 2.5x and 5x (50x total magnification), except for the lateral boundary, which was identified by switching through the 2.5x, 5x and 10x (100x total magnification) objectives.

For all brains, we aimed to use sections as close to every 20^{th} section as possible by selecting the best quality series to be our main series (i.e. series where majority of sections

originated); however, due to the fragile nature of our nestling tissue, all brains had some damaged or missing sections. Whenever a section was damaged or missing, we used an adjacent section from the previous or subsequent series (see Table S1). If no adjacent section was available, we used the next closest available non-adjacent section (see Table S1). If no section was available, we used the "Account for missing section" function, which averages the volume from the previous and subsequent section and substitutes that as the volume for the missing section (see Table S1). Out of 24 brains, 6 early age class brains had at least one missing section and 3 late age class brains had at least one missing section.

We measured hippocampal volume with the Cavalieri estimator method in the Stereo Investigator (MBF Bioscience) and the DAPI channel to measure hippocampal volume with the previously drawn boundaries (Fig. 2b). For early age class brains, we used a grid size of 105 µm x 105 µm and for late age class brains, we used a grid size of 195 µm x 195 µm. For all brains, we set the interval to be equal to every 20th. We only marked the grid points whenever it was clear that the tissue belonged to the hippocampus. Whenever a section was folded over, or torn, we marked extra grid points to estimate the folded/missing area. When the two hemispheres were touching, we inspected the green-fluorescent protein (GFP) channel, which displayed tissue structure, to determine whether we could see connective tissue between sections, overlapping sections, or a separation between sections. As with the telencephalon volume measurements, we completed all hippocampal boundary mapping and volume measurements blind to dosing conditions, but not age. For individuals with a missing section, we present the estimate adjusted with missing section volume. A Gunderson Coefficient of Error $(CE)_{m=1}$ of 0.01 to 0.022 for early brains and of 0.009 and 0.012 for late brains was achieved.

2.8. Hippocampal cell proliferation quantification

We counted EdU^+ cells (i.e. cells that incorporated EdU between EdU injection and brain collected and were stained for EdU) for measurements of cell proliferation. We utilized the same sections for hippocampal volume and EdU^+ cell counting, meaning the boundaries were identical, the section interval remained constant, and the number of adjacent, non-adjacent and missing sections were the same for both (see Table S1).

We used the Optical Fractionator method in the Stereo Investigator (MBF Bioscience) and the Cy5 channel to visualize EdU^+ cells (Fig. 3b). The grid size was optimized using one brain per treatment group and age class, for a total of four brains. For optimization, we counted all cells within the hippocampus by setting the counting frame equal to grid size (175 μm x 175 μm) (counting details below). Then, for the two brains in the same age class, we used the "resample oversample" tool in Stereo Investigator (MBF Bioscience) and plotted the nestlings total EdU^+ cells count estimation by fold increase in sites. With this, we identified the optimal fold increase in size where both nestlings total EdU^+ cell count estimation were not overlapping. This number (e.g. 2-fold increase) was then used to calculate the new-grid size for counting cells within the specific age class. We chose to use a final grid size of 300 μm x 300 μm for early age class brains and of 250 μm x 250 μm for late age class brains, while keeping the counting frame at 175 μm x 175 μm for both.

With optimized parameters, we counted the remainder of the brains included in the study, apart from those that had missing sections (sample sizes: Control: early: n = 2; late: n = 4; CPF: early: n = 3; late: n = 5). Data for the remaining brains will be collected in the future. EdU^+ cells were counted within the z-axis of the left hemisphere hippocampus at 63x (630x total magnification) objective. First, the thickness of the site was quantified using the fine focus of the

microscope to define the immediate defocus at the top and bottom of the section. For each section, site thickness was measured every 3 sites and every site with EdU^+ cells within the hippocampus. EdU^+ cells were marked only when the top came into focus within the counting frame. During optimization and counting, we did not use guard zones due to the thin tissue thickness, thus, we set it to be equal to 0 µm, with a disector equal to pre-staining section thickness (i.e. 20 µm). However, after counting, we used the "resample dissector" function in the Stereo Investigator (MBF Bioscience) which provided us with cell estimates with a dissector the height equal to the minimum measured site and mean of all measured sites. Due to the relatively high number sites that were thinner than the mean measured sites, we used the estimate with a disector height equal to the minimum measured site and a top guard zone of 0 µm. Disector heights ranged from 5.6 to 6.3 µm for early age class brains and 5.2 to 6.7 µm for late age class brains. Because of the wavy thickness and uneven neuron distribution in the sections, we present the total number of neurons based on the number-weighted section thickness estimate. As with the telencephalon and hippocampal volume measurements, all hippocampal cell counting was done blind to dosing conditions, but not age. The Gunderson $CE_{m=1}$ ranged between 0.038 and 0.049 for early age class brains and between 0.056 and 0.099 for late age class brains.

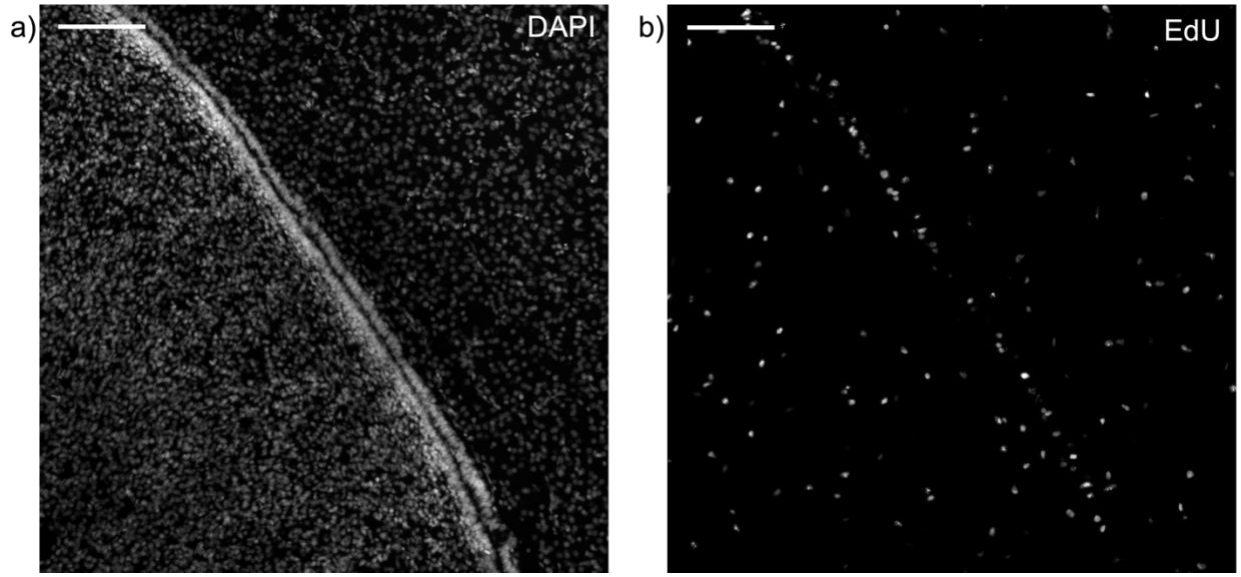

Fig. 3: Examples of a) 4′,6-diamidino-2-phenylin-dole (DAPI) and b) 5-ethynyl-2'-deoxyuridine (EdU) staining. The hippocampus is in the right half of each image and the telencephalon is in the left half of each image, with the ventricular zone separating each region. Scale bar = 100 μm (a and b).

2.9. Statistical analysis

All statistical analysis were completed in R and RStudio (version # 4.3.1 for R and RStudio) (R Core Team, 2023). We completed linear mixed effects models using the *lmer* function from the lmerTest package for all analyses (Kuznetsova et al., 2017). Level of significance was set to be $p < 0.05$. Results for all models are presented using the *anova* function from the stats package which provided a type II analysis of variance (ANOVA) with Satterthwaite's method output (R Core Team, 2023). For all significant interactions, we completed a post-hoc test. We first used the *emmeans* function from the emmeans package to calculate the estimated marginal means of the interaction (Lenth, 2023). Then, we used the *pairs* function from the emmeans package to perform simple pairwise comparisons for each factor in

the interaction (Lenth, 2023). All plots depicting results were made using the ggplot2 package (Wickham, 2016).

For all validations of linear mixed effects models, we checked for normality of model residuals by visually inspecting QQ-plots of model residuals and using the *shapiro.test* function from the stats package (R Core Team, 2023). We also checked for homoscedasticity of residuals by inspecting homoscedasticity plots. QQ-plots and homogeneity plots are presented in supplementary materials (Fig. S1-S4). Finally, we checked for autocorrelation using the *acf* function from the stats package (R Core Team, 2023).

For ChE and body mass analyses, some nestlings that were initially included in the study did not survive until the brain collection period due to predation or death. Therefore, we did not have sex information for those individuals, and they were excluded from analyses, as sex had significant effects on these responses. Moreover, we removed ChE data and body mass data from any individuals who were used as test individuals. Unlike analyses for ChE activity and body mass, we did not include sex in our model on hippocampal volume and cell proliferation due to low sample sizes.

2.9.1. ChE activity

We analyzed square-root transformed ChE activity with a linear mixed effects model. Our fixed effects were the treatment group (2 levels = control, chlorpyrifos) by sex (2 levels = female, male) interaction. Our random effect was nest ID, to control for nest effects. The dataset included one nestling whose blood sample was taken 25 hours after dosing which was ~9 hours later than typical sampling. We redid the analysis without that nestling and obtained the same results; therefore, we retained this sample in the statistical analysis.

2.9.2. Body mass

We analyzed raw body mass data with a linear mixed effects model. Our fixed effects were the treatment group (2 levels = control, chlorpyrifos) by age class (5 levels = dosing, blood sampling, early, middle, late) interaction, treatment group by sex (2 levels = female, male) interaction and age class by sex interaction. Our random effects were nest ID, to control for nest effects, and bird ID, to control for repeated measures. We recognize that the issues with normality, homogeneity and autocorrelation are a limitation (Fig. S2).

2.9.3. Hippocampal volume

We analyzed corrected hippocampal volume with a linear mixed effects model. To obtain a response variable that corrects for allometric effects of telencephalon volume on hippocampal volume, we used residuals from a linear model of raw hippocampal volume against raw telencephalon volume minus hippocampal volume. The hippocampal volume corrected for telencephalon volume will be referred to as the "corrected hippocampal volume" hereafter. Our fixed effects were the treatment group (2 levels = control, chlorpyrifos) by age class (2 levels = early, late) interaction. Our random effect was nest ID, to control for nest effects.

2.9.4. Hippocampal cell proliferation

We analyzed the corrected number of EdU^+ cells with a linear mixed effects model. To obtain a response variable that accounts for the possibility of a larger hippocampal having more cells, we used residuals from a linear model of the total number of EdU^+ cells against raw hippocampal volume. The number of EdU^+ cells corrected for hippocampal volume will be

referred to as the "corrected number of EdU^+ cells" hereafter. Our fixed effects were the treatment group (2 levels = control, chlorpyrifos) by age class (2 levels = early, late) interaction. Our random effect was nest ID, to control for nest effects.

3. Results

3.1. ChE activity

ChE activity was significantly affected by treatment group ($F_{1, 54.98} = 111.31$, $p < 0.001$), with ChE activity being lower in CPF-dosed nestlings relative to control nestlings (Fig. 4). ChE activity was not significantly affected by sex ($F_{1, 60.33} = 2.61$, $p = 0.11$); however, it was significantly affected by the treatment group by sex interaction ($F_{1, 57.10} = 5.20$, $p = 0.026$) (Fig. 4). In both sexes, ChE activity was significantly different between control and CPF-dosed nestlings (*post-hoc* $p < 0.0001$ for each sex), with ChE activity being lower in CPF-dosed nestlings relative to control nestlings. Moreover, ChE activity was significantly different between female and male CPF-dosed nestlings (*post-hoc* $p = 0.013$), with males being more affected than females (Fig. 4). However, it was not significantly different in female and male control nestlings (*post-hoc* $p = 0.066$).

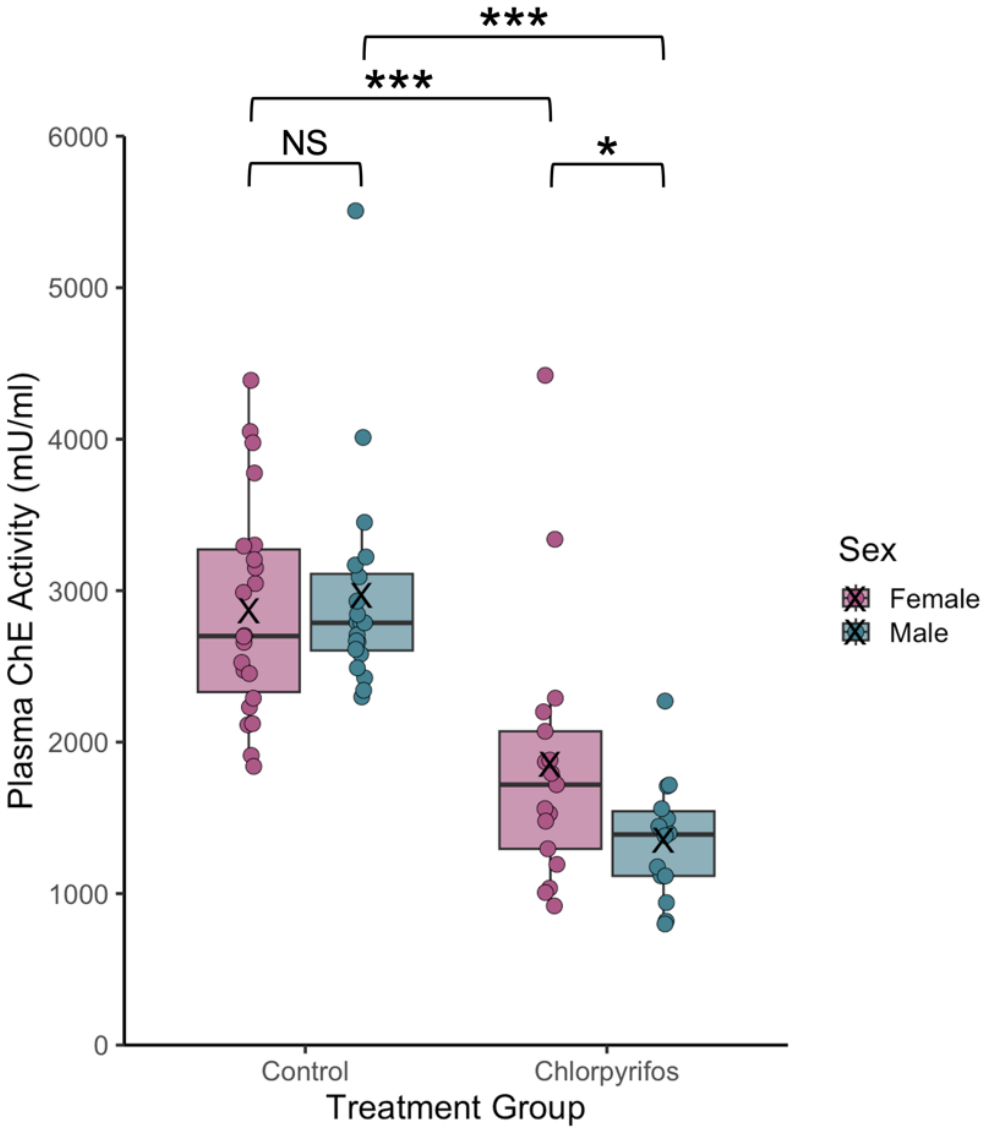

Fig. 4: Plasma cholinesterase (ChE) activity (mU/ml) in blood samples taken from female and male nestling European starlings the day after exposure (16 hours post-dosing, PHD 2-4) to either a control or chlorpyrifos (CPF) dose. Boxplots indicate 25th, 50th, and 75th percentiles, horizontal lines indicate the median and whiskers indicate range, with individual jittered datapoints points overlaid. Boxplot colour indicates sex: female (pink), male (blue). 'X' indicates mean ChE activity. Asterisks indicate significant differences between groups (***: $p < 0.001$; *: $p < 0.05$) and NS (i.e. not significant) indicates no significant difference between groups. Note

that raw data is plotted in graph; however, ChE activity was square-root transformed for statistical analysis.

3.2. Body mass

Body mass was not significantly affected by treatment group ($F_{1, 65.40} = 0.36$, $p = 0.55$); however, it was affected by age class ($F_{4, 201.41} = 2011.85$, $p < 0.001$) and sex ($F_{1, 72.80} = 12.46$, $p < 0.001$). Body mass increased with age between the blood sampling and late age classes and was higher in males relative to females (Fig. 5). Body mass was not significantly affected by the treatment group by age class interaction ($F_{4, 203.67} = 1.71$, $p = 0.15$) or the treatment group by sex interaction ($F_{1, 60.76} = 0.18$, $p = 0.68$). However, it was significantly affected by the age class by sex interaction ($F_{4, 202.46} = 3.76$, $p = 0.006$) (Fig. 5). Body mass between females and males in the early age class (*post-hoc* $p = 0.0041$), middle age class (*post-hoc* $p = 0.0006$) and late age class (*post-hoc* $p = 0.002$) was significantly different, where body mass was higher in males in all three age classes (Fig. 5). Body mass in females was significantly different between the blood sampling and early age class (*post-hoc* $p < 0.0001$), early and middle age classes (*post-hoc* $p < 0.0001$) and middle and late age classes (*post-hoc* $p = 0.006$). Moreover, body mass in males was significantly different between the blood sampling and early age class (*post-hoc* $p < 0.0001$), early and middle age classes (*post-hoc* $p < 0.0001$) and middle and late age classes (*post-hoc* $p < 0.001$). In both sexes, body mass increased between each age class (Fig. 5).

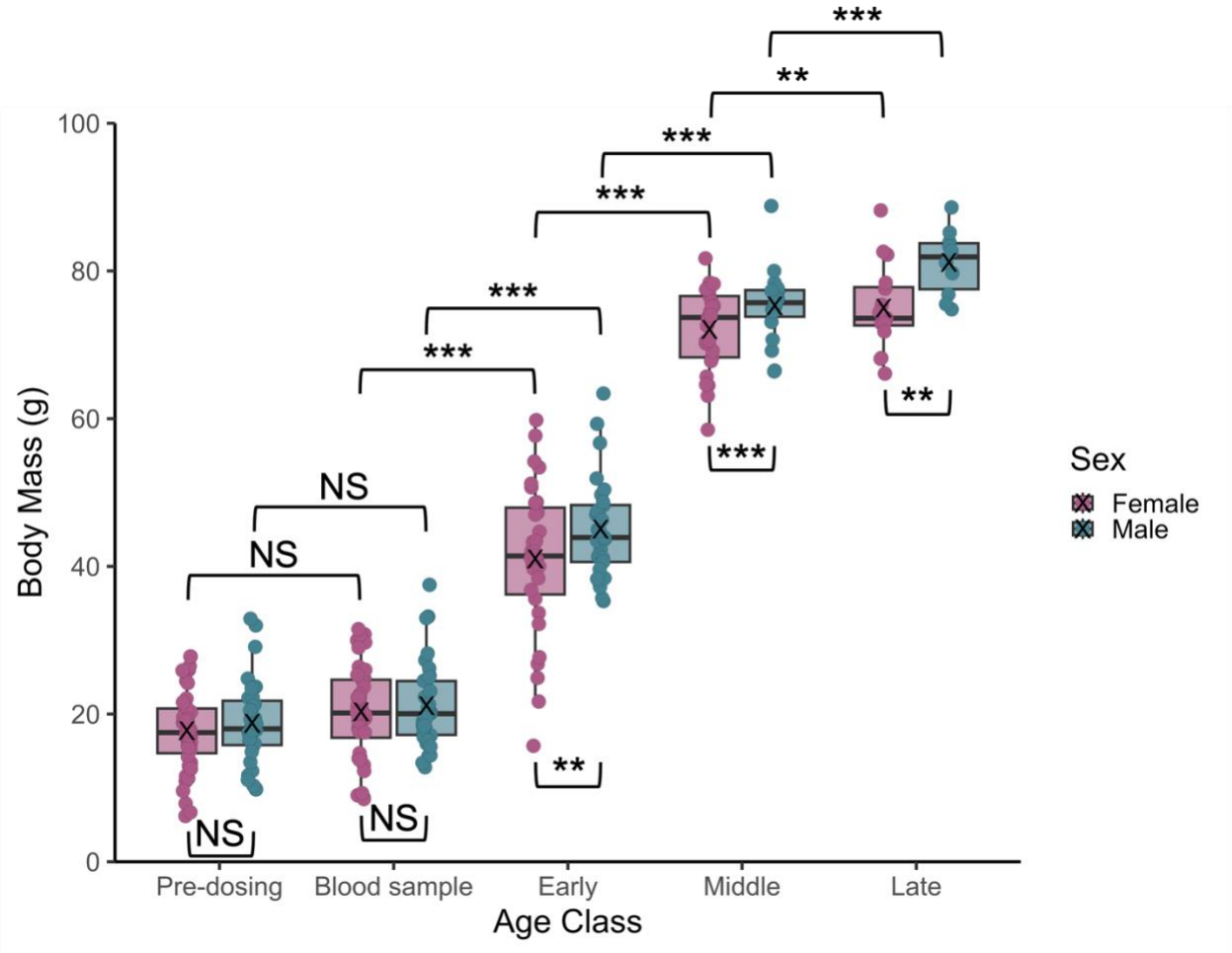

Fig. 5: Body mass (g) measured immediately pre-dosing (post-hatch day (PHD) 1-3), on day of blood sampling (16 hours post-dosing, PHD 2-4), early age class (4 days post-dosing, PHD 5-7), middle age class (10 days post-dosing, PHD 11-13) and late age class (18 days post-dosing, PHD 19-20) in nestling European starlings after exposure to either a control or chlorpyrifos (CPF) dose. Boxplots indicate 25th, 50th, and 75th percentiles, horizontal lines indicate the median and whiskers indicate range, with individual jittered datapoints points overlaid. Boxplot colour indicates sex: female (pink), male (blue). X indicates mean body mass. Asterisks indicate significant differences between groups (***: $p < 0.001$; **: $p < 0.01$) and NS (i.e. not significant) indicates no significant difference between groups. Treatment group was not included in the figure because it did not significantly affect body mass.

3.3. Hippocampal volume

Corrected hippocampal volume was not significantly affected by treatment group ($F_{1, 12.70}$ = 1.17, p = 0.30) or age class ($F_{1, 16.92}$ = 0.046, p = 0.83). However, it was significantly affected by the treatment group by age class interaction ($F_{1, 12.70}$ = 6.08, p = 0.029). Corrected hippocampal volume was significantly different between control and CPF-dosed nestlings in the late age class (*post-hoc* p = 0.031), with it being larger in CPF-dosed nestlings relative to control nestlings (Fig. 6). However, corrected hippocampal volume was not significantly different between the control and CPF-dosed nestlings in the early age class (*post-hoc* p = 0.41) (Fig. 6). Moreover, corrected hippocampal volume was not significantly different across age classes in control nestlings (*post-hoc* p = 0.1) or in CPF-dosed nestlings (*post-hoc* p = 0.22) (Fig. 6).

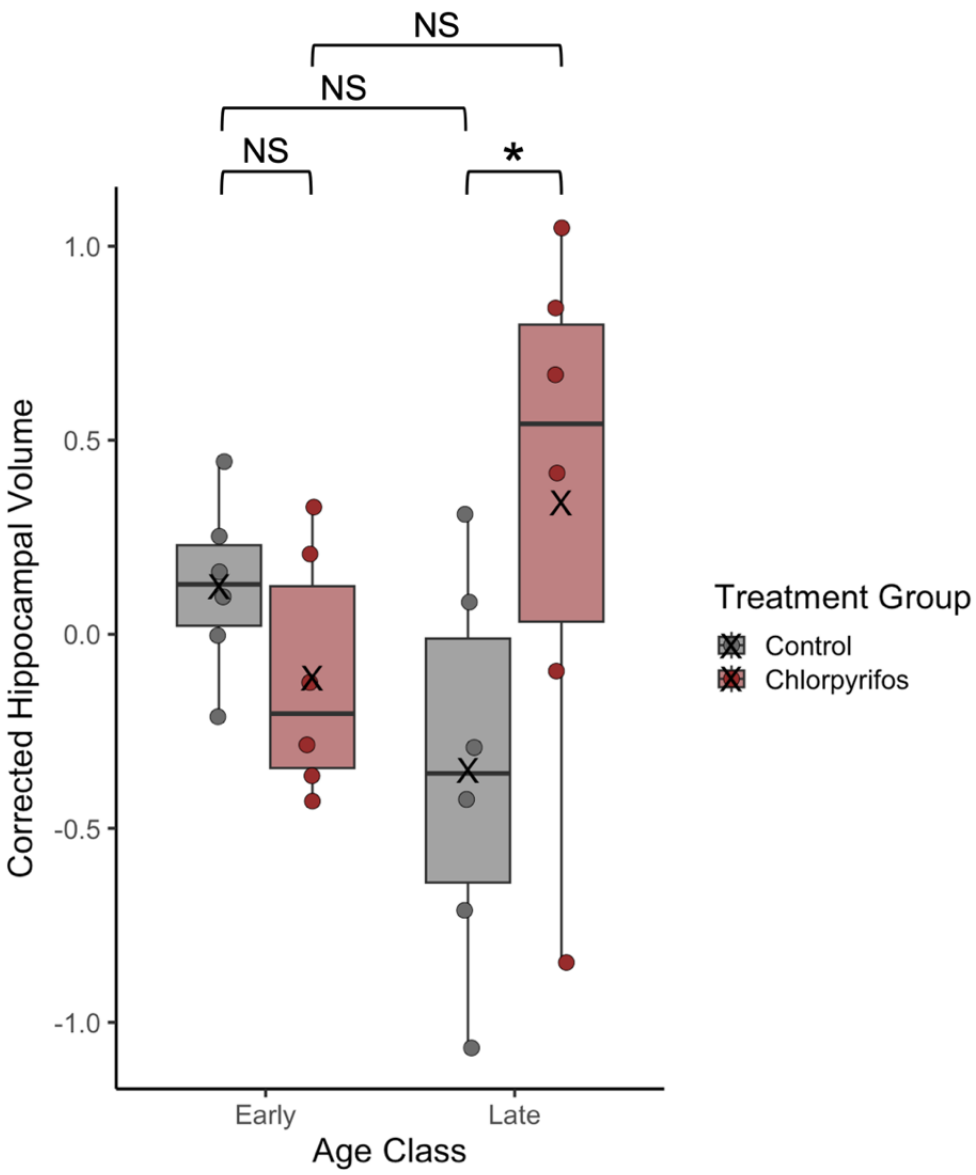

Fig. 6: Corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume) in nestling European starling brains collected on the early age class (post-hatch day (PHD) 5-7) and late age class (PHD 19-20) European starling nestlings after exposure to either a control or chlorpyrifos (CPF) dose. Boxplots indicate 25th, 50th, and 75th percentiles, horizontal lines indicate the median and whiskers indicate range, with individual jittered datapoints points overlaid. Boxplot colour indicates treatment groups: control (grey), CPF (red). 'X' indicates

mean corrected hippocampal volume. Asterisks indicate significant differences between groups (*: $p < 0.05$) and NS (i.e. not significant) indicates no significant difference between groups.

3.4. Cell proliferation

The corrected number of EdU^+ cells was not affected by treatment group ($F_{1, 9.19} = 0.93$, $p = 0.36$), or age class ($F_{1, 8.99} = 2.20$, $p = 0.17$). However, it was affected by the treatment group by age class interaction ($F_{1, 9.19} = 7.26$, $p = 0.024$). The corrected number of EdU^+ cells was significantly different across age classes in control nestlings (*post-hoc* $p = 0.02$); with the corrected number of EdU^+ cells decreasing between the early and late age classes (Fig. 7). However, the corrected number of EdU^+ cells was not significantly different across age classes in the CPF-dosed nestlings (*post-hoc* $p = 0.59$). Moreover, the corrected number of EdU^+ cells was not significantly different between control and CPF-dosed nestlings in the early age class (*post-hoc* $p = 0.10$) or the late age class (*post-hoc* $p = 0.079$).

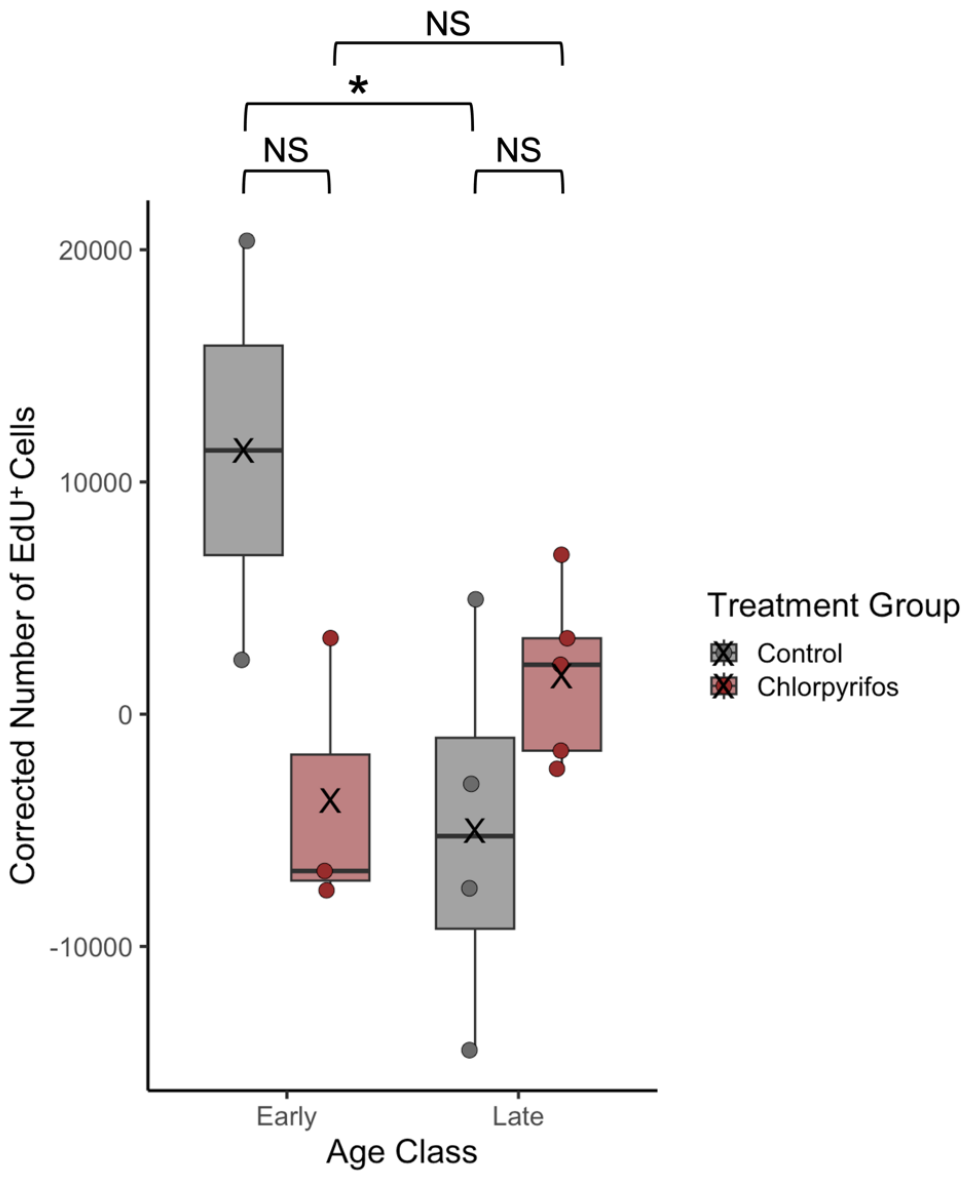

Fig. 7: Corrected number of 5-ethynyl-2'-deoxyuridine $(EdU)^+$ cells (i.e. number of EdU^+ cells corrected for hippocampal volume) in the hippocampus of nestling European starling brains collected on the early age class (post-hatch day (PHD) 5-7) and late age class (PHD 19-20) after exposure to either a control or chlorpyrifos (CPF) dose. Boxplots indicate 25th, 50th, and 75th percentiles, horizontal lines indicate the median and whiskers indicate range, with individual jittered datapoints points overlaid. Boxplot colour indicates treatment groups: control (grey), CPF (red). X indicates mean corrected number of EdU^+ cells. Asterisks indicate significant

differences between groups (*: $p < 0.05$) and NS (i.e. not significant) indicates no significant difference between groups.

4. Discussion

In this study, we dosed developing free-living starling nestlings shortly after hatching (i.e. PHD 1-3) with an acute sublethal dose of 6 mg/kg-bw and examined both developmental changes in the hippocampus and the effects of CPF exposure on body mass and the hippocampus. We found both developmental changes in the corrected number of EdU^+ cells and an effect of CPF exposure on corrected hippocampal volume and the corrected number of EdU^+ cells. Overall, our results suggest that an early acute sublethal dose of CPF after hatching can directly alter normal hippocampal development patterns. This is of concern because any alteration in normal hippocampal developmental patterns could potentially have long-term effects on fitness-related behaviours and cognitive traits.

4.1. ChE activity

We exposed nestlings to a single dose of CPF and aimed for our dosing concentration to be sublethal but of toxicological relevance. We achieved this effect, as confirmed with ChE activity, which was lower in CPF-dosed nestlings relative to control nestlings (Fig. 4). Our target dose of 6.0 mg/kg-bw falls within the range of doses that was reported to decrease 50% of brain ChE activity in starlings (Meyers et al., 1992). However, our exposure dose was higher than 2.5 mg/kg which resulted in the death of one third of nestlings (Meyers et al., 1992). Although previously reported LD50 for CPF in starlings has large variation (Schafer, 1972; reviewed in Schafer & Brunton, 1979), we were able to sublethal effects with a dose of 6.0 mg/kg-bw.

The sex effect on ChE activity we observed is consistent with other studies that have observed sex differences in ChE inhibition after CPF exposure. For example, male rats had higher ChE inhibition in all brain regions that were measured two hours after CPF exposure on PND 1 compared to females (Dam et al., 2000). Contrarily, male rats exposed to CPF had less ChE inhibition in the brain compared to females when exposed as adults; however, ChE inhibition was similar when dosed on PND 17 and PND 27 (Moser et al., 1998). In sum, our CPF treatment was effective, and tended to have a stronger effect on males than females.

4.2. Nestling growth and the effects of CPF exposure

Post-hatching growth in altricial species is characterized by rapid growth (O'Connor, 1977); however, CPF exposure can affect body mass (Ahmad et al., 2015), although there is also evidence of recovery (Richards et al., 2000). Moreover, early nutritional stress can have effects on the hippocampus (Pravosudov et al., 2005), thus, any effects CPF may have on body mass may later affect hippocampus. In the present study, we aimed to determine whether effects of CPF exposure on the brain were a direct effect of exposure, or indirect effect of exposure due to early nutritional stress. We showed that starlings gained mass with across age classes; however, CPF exposure did not have any effects on developmental changes in body mass at any age classes (Fig 5). These results are not consistent with other studies that showed effects on body mass and recovery after OP exposure (e.g. dicrotophos, famphur) in birds. For example, repeated CPF exposure decreased broiler chicks body mass during certain weeks of the experiment compared to controls (Ahmad et al., 2015). Starling nestlings dosed with CPF (Richards et al., 2000), dicrotophos (Stromborg et al., 1988) and famphur (Powell & Gray, 1980) had lower body mass than controls. Moreover, starling nestlings dosed with dicrotophos (Stromborg et al., 1988)

and with CPF (Richards et al., 2000) showed recovery in body mass. In our study, CPF had a strong and clear effect on the brain (see below), without affecting growth, indicating that our reported effects are not due to a generalized effect on overall health and early nutritional stress (e.g. food consumption, nausea, etc.).

4.3. Effects of CPF exposure on developmental changes in hippocampal volume

The hippocampus is a brain region significant for migratory behaviour (Carvalho-Paulo et al., 2018; Cristol et al., 2003; LaDage et al., 2011; Pravosudov et al., 2006). In the present study, we aimed to look at whether there are developmental changes in corrected hippocampal volume at two age classes in the nestling period; however, we did not observe any significant changes in corrected hippocampal volume across age classes in control nestlings (Fig. 6). This is inconsistent with what was found in chicks, where hippocampal volume increased significantly between PHD 1 and PHD 7 (Sahin et al., 2002). However, this discrepancy between our results and those of Sahin et al. (2002) may be due to species differences, age differences or methodological differences.

We also aimed to determine whether CPF exposure affected corrected hippocampal volume. We detected that corrected hippocampal volume in the late age class was larger in CPF-dosed nestlings relative to control nestlings (Fig. 6). These results are inconsistent with a study that showed no effect of CPF exposure on hippocampal volume in guinea pigs (*Cavia porcellus*) (Mullins et al., 2013) and another that found that no effect of CPF exposure on the size of certain brain areas in zebrafish (*Danio rerio*) (Richendrfer & Creton, 2015). However, these results are different to those that found the hindbrain and forebrain size decreased in zebrafish after malathion exposure, another OP insecticide (Richendrfer & Creton, 2015). Again, these

discrepancies may be due to species differences, age differences and methodological differences. Nonetheless, these results show that a single dose of CPF early in the nestling period can affect corrected hippocampal volume late in the nestling period.

The larger corrected hippocampal volume that we observed in the late age class CPF-dosed nestlings relative to control nestlings could be explained by a compensatory production of glial cells or compensatory production of cells due to apoptosis. An increase in the number of brain cells of different sizes is a major contributor to the growth of the brain (Bandeira et al., 2009). After exposure to chemicals, glial cells expressing glial fibrillary acidic protein (GFAP) can increase as a response (reviewed in O'Callaghan, 1988). One study has shown that CPF exposure increased the number of glial cells (Garcia et al., 2002). Moreover, CPF exposure can cause apoptosis (Caughlan et al., 2004; Gupta et al., 2010), which has been demonstrated to induce compensatory cell proliferation (Ryoo et al., 2004). Thus, although we did not label glial cells or apoptotic cells, CPF exposure may have induced compensatory cell production, which contributed to the larger corrected hippocampal volume in the late age class CPF-dosed nestlings.

4.4. Effects of CPF exposure on developmental changes in hippocampal cell proliferation

Hippocampal cell proliferation, the production of new cells, is a fine-scale measure that is associated with spatial cognition in birds (Patel et al., 1997). We used EdU, an exogenous marker of cell proliferation, and aimed to detect whether there are changes in the corrected number of EdU^+ cells at two age classes in the nestling period. We found that the corrected number of EdU^+ cells decreased between the early and late age classes in control nestlings (Fig. 7). Rates of the cell cycle can differ depending on age of the individual and differ depending on

the species examined (Charvet & Striedter, 2008) and to our knowledge, no study has examined cell proliferation in starling nestlings or other closely related species. However, our results are consistent with other studies that show that the production of new brain cells decreases with age. For example, over a longer timescale, the density of proliferating cell nuclear antigen $(PCNA)^+$ cells, an endogenous marker of cell proliferation, and doublecortin $(DCX)^+$ cells, an endogenous marker of neurogenesis, decreased in many regions of the brain in quail (*Cortunix japonica*) after hatching (Nkomozepi et al., 2018). DCX^+ labelling also decreased in some brain regions of domestic chicks after hatching (Mezey et al., 2012). In zebra finch (*Taeniopygia guttata*), a songbird, the proliferative zone decreases in size by the first week after hatching, meaning cell proliferation occurs earlier on during development (i.e. during the embryo stages and the first week of hatching) in zebra finch (Charvet & Striedter, 2009). Although we had low sample sizes, our results clearly show a decrease in the corrected number of EdU^+ cells over the nestling period in an altricial bird.

We also aimed to determine whether CPF exposure has any effect on developmental changes in the corrected number of EdU^+ cells. Unlike what we found with control nestlings, in CPF-dosed nestlings, there was a lack of a decrease in the corrected number of EdU^+ cells across age classes (Fig. 7). These results do not match with our prediction and they are inconsistent with studies showing decreased DNA synthesis and protein synthesis (Whitney et al., 1995), and decreased hippocampal proliferating cells with subsequent recovery (Ohishi et al., 2013) after CPF exposure. However, as with corrected hippocampal volume, the absence of a decrease in the corrected number of EdU^+ cells may be due to compensatory cell production. Cell proliferation may have continued in late age class at a level similar to the early age class due to either an increase in glial cells (Garcia et al., 2002; reviewed in O'Callaghan, 1988), or due to apoptosis

(Caughlan et al., 2004; Gupta et al., 2010; Ryoo et al., 2004). Thus, the lack of a decrease in the corrected number of EdU^+ cells across age classes may be explained by compensatory cell production.

The results on the corrected number of EdU^+ cells we found in CPF-dosed nestlings may also be related to the larger corrected hippocampal volume we observed in CPF-dosed nestlings in the late age class. To the best of our knowledge, the timeline explaining how fast hippocampal volume can increase after new cells are produced is not known; however, other studies may provide insight. In domestic chicks, cells labeled with BrdU, another exogenous marker of cell proliferation, were also labelled with DCX, an endogenous marker expressed in immature migrating neurons, the day after individuals were injected with BrdU (Mezey et al., 2012). Furthermore, in adult birds, after injection of [^{3}H]-thymidine, another exogenous marker of cell proliferation, cells begin migrating from the ventricular zone within three days after injection, and can reach the lateral telencephalon within 20 days after injection (Alvarez-Buylla & Nottebohm, 1988). Summed together, as early as one after a new cell is produced, it may begin migrating to its final destination. The, the lack of a change in the corrected number of EdU^+ cells across age classes may thus explain the larger corrected hippocampal volume in the late age class in CPF-dosed nestlings relative to control nestlings.

5. Conclusions and future directions

Overall, to our knowledge, this is the first study examining the effect of acute OP exposure on the hippocampus in a migratory songbird species during the nestling period. We found both developmental changes and effects of CPF exposure on the hippocampus. Future work should examine neural and glial markers and apoptosis to determine more specifically

whether specific cell types are targeted by CPF exposure. Linking neural effects during development to long-term behavioural effects, such as on spatial cognition and migratory ability, will be important to determining whether effects seen in the hippocampus do result in behavioural deficits later in life. This can be performed with a spatial memory test during adulthood. Contrarily, examining how CPF exposure may affect non-migratory populations of starlings in addition to other brain regions is important, because although alterations in normal hippocampal development may only affect fitness in migratory starlings, CPF exposure may affect other brain regions more significant for fitness, especially in non-migrants, such as the song control nuclei. Furthermore, more doses of CPF can be included to determine exactly what dose causes a change in cell proliferation and what doses have no effects. We might expect that higher doses may have a stronger effect on hippocampal volume and cell proliferation; however, a lower dose may have a more negligible effect. Additionally, increasing sample sizes is important to determine whether the sex-related effects observed on ChE activity are also observed in the brain. Overall, although much research needs to be done, the results in this study further our understanding of hippocampal development within a migratory songbird and how exposure to a neurotoxic insecticide can alter normal developmental processes.

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Credit authorship statement

Sereena Moore: Investigation, Methodology, Formal analysis, Writing – Original draft, Visualization, Funding acquisition. **Claire Bottini:** Investigation, Methodology, Assistance with formal analysis, Writing – Reviewing and editing. **Margaret Eng:** Conceptualization, Investigation, Resources, Methodology, Assistance with formal analysis, Writing – Reviewing and editing, Supervision, Funding acquisition. **Christy Morrissey:** Conceptualization, Investigation, Resources, Methodology, Assistance with formal analysis, Writing – Reviewing and editing, Supervision, Funding acquisition. **Mélanie Guigueno:** Conceptualization, Resources, Methodology, Assistance with formal analysis, Writing – Reviewing and editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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Data availability

The data used in the article can be provided if requested.

Supplementary information

Table S1: Minimum and maximum number of total sections, main sections (i.e. series where majority of sections come from for a given brain), adjacent sections, non-adjacent sections and missing sections used for corrected hippocampal volume measurements in early and late age class brains.

	Total sections		Main sections		Adjacent sections		Non-adjacent sections		Missing sections	
Age class	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
Early	9	12	6	9	0	3	0	2	0	2
Late	25	15	11	20	0	6	0	5	0	4

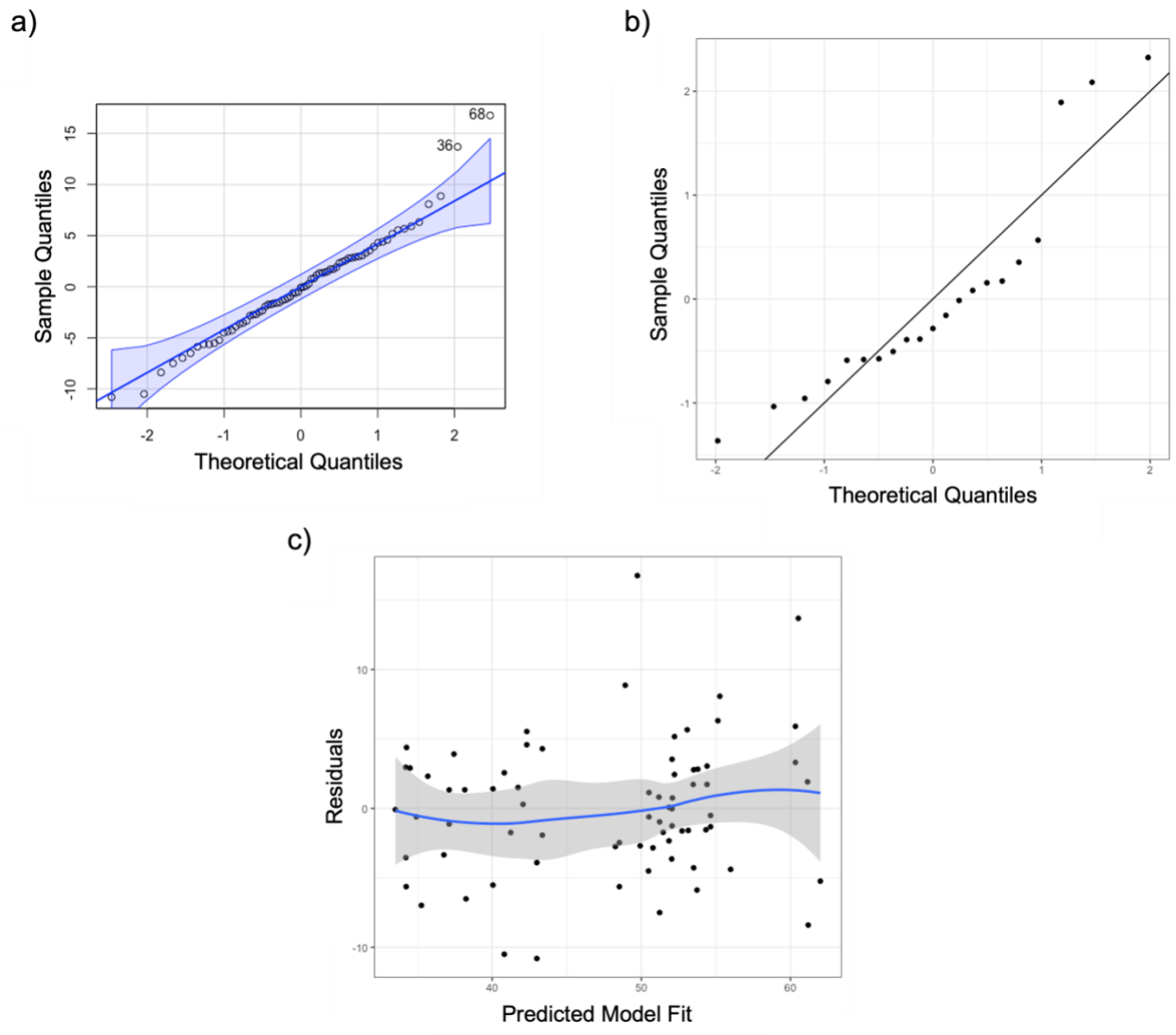

Fig. S1: Diagnostic plots used to validate the linear mixed effects model analyzing the effect of the treatment group by sex interaction on square-root transformed ChE activity (mU/ml) with a random effect of nest ID. a) Quantile-quantile plot showing distribution of model residuals used to check for residual normality (*Shapiro-Wilk test p* = 0.12). b) Quantile-quantile plot showing distribution of random effect (nest ID) residuals used to check for residual normality. c) Plot of residuals versus fitted values used to check for homogeneity.

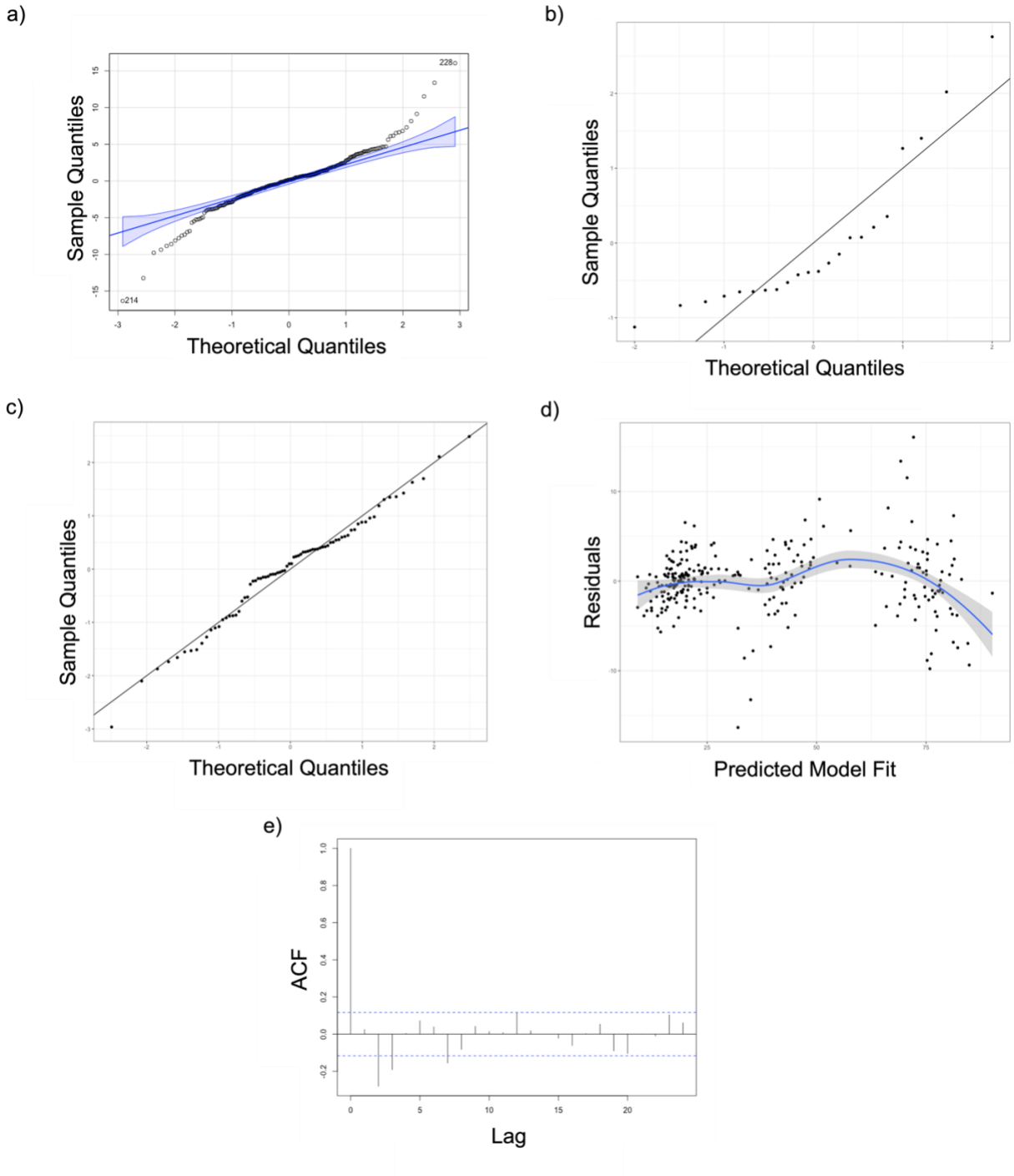

Fig. S2: Diagnostic plots used to validate the linear mixed effects model analyzing the effect of the treatment group by age class, treatment group by sex and age class by sex interactions on body mass (g) with a random effect of nest ID. a) Quantile-quantile plot showing distribution of model residuals used to check for residual normality (*Shapiro-Wilk test p* = 0.12). b) Quantile-

quantile plot showing distribution of random effect (nest box ID) residuals used to check for normality. c) Quantile-quantile plot showing distribution of random effect (bird ID) residuals used to check for normality. d) Plot of residuals versus fitted values used to check for homogeneity. e) Autocorrelation plot used to check for autocorrelation.

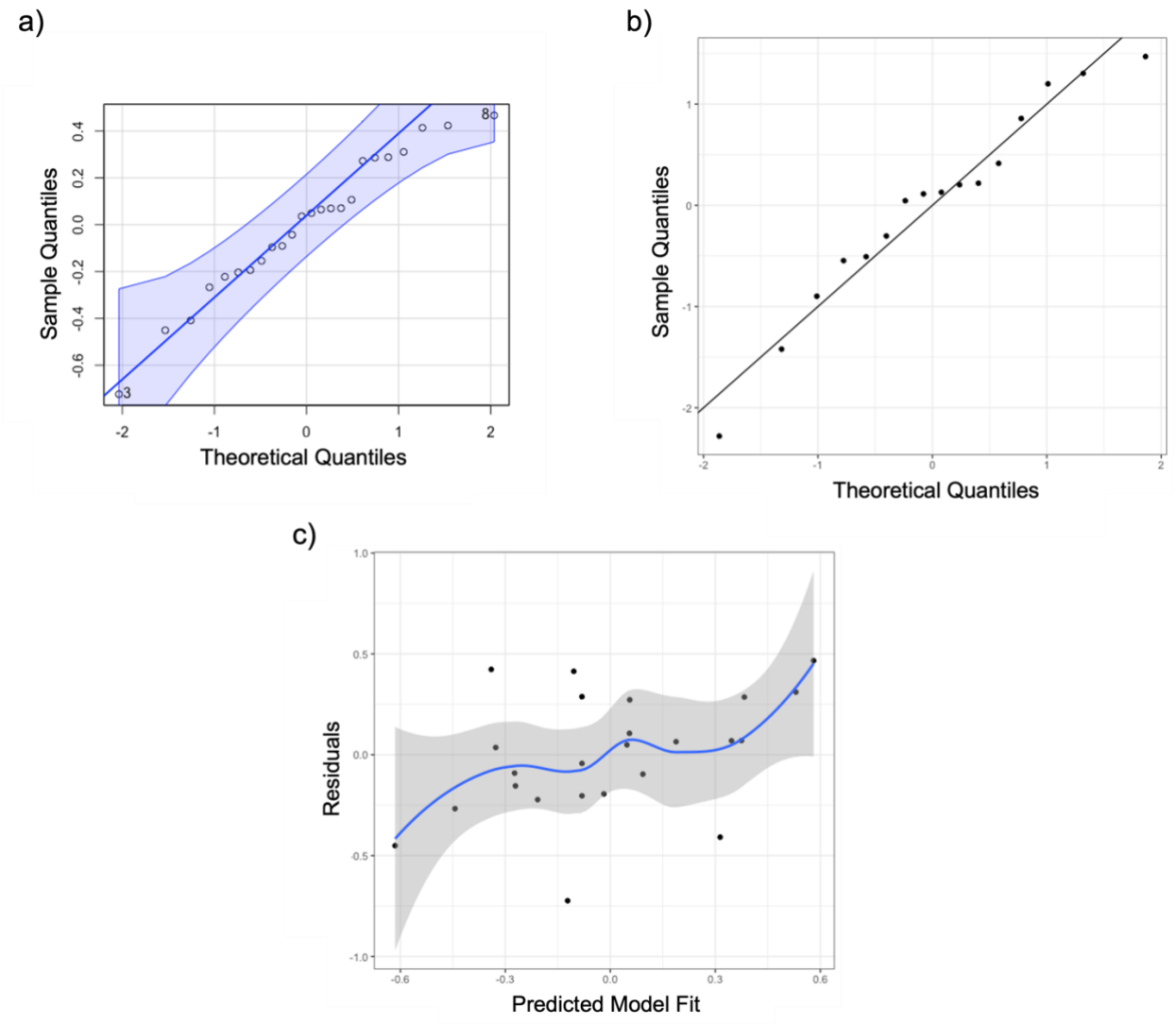

Fig. S3: Diagnostic plots used to validate the linear mixed effects model analyzing the effect of the treatment group and age class interaction on corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume) with a random effect of nest ID. a) Quantile-quantile plot showing distribution of model residuals used to check for residual normality (*Shapiro-Wilk test p* = 0.61). b) Quantile-quantile plot showing distribution of random effect (nest ID) residuals used to check for residual normality. c) Plot of residuals versus fitted values used to check for homogeneity.

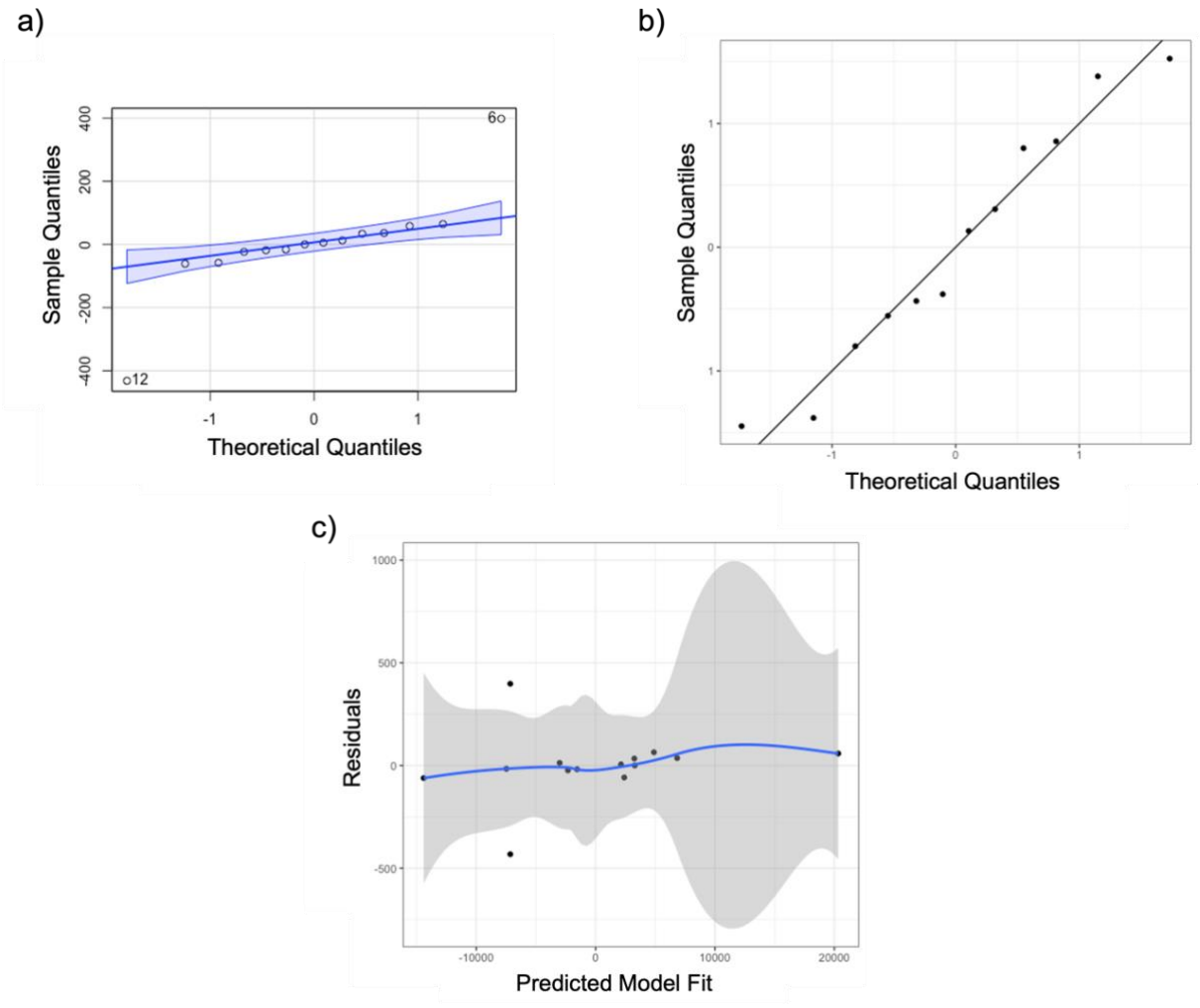

Fig. S4: Diagnostic plots used to validate the linear mixed effects model analyzing the effect of the treatment group by age class interaction on the corrected number of EdU^+ cells (i.e. number of EdU^+ cells corrected for hippocampal volume) with a random effect of nest ID. a) Quantile-quantile plot showing distribution of model residuals used to check for residual normality (*Shapiro-Wilk test p* = 0.0017). b) Quantile-quantile plot showing distribution of random effect (nest ID) residuals used to check for residual normality. c) Plot of residuals versus fitted values used to check for homogeneity.

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CHAPTER 3: GENERAL DISCUSSION

Thesis summary

Normal developmental patterns can be adversely affected by developmental exposure to stressors in the environment. However, there are limited data that examine developmental changes, at both large- and fine-level scales, in the hippocampus of wild birds, and whether developmental exposure to a neurotoxic insecticide can adversely affect normal hippocampal development. As such, in this thesis, I 1) examined developmental changes in a large-scale (volume) and a fine-scale (cell proliferation) measure in the hippocampus during post-hatching nestling development in a migratory songbird, the European starling (*Sturnus vulgaris*) (hereafter "starlings") and 2) examined whether early acute sublethal exposure to chlorpyrifos (CPF) disrupts developmental changes in body mass and the hippocampus during post-hatching nestling development. In chapter 2 of this thesis, I found both developmental changes and effects of CPF exposure on the hippocampus.

First, I examined developmental changes in the hippocampus. I examined corrected hippocampal volume (i.e. hippocampal volume corrected for telencephalon volume) and the corrected number of 5-ethynyl-2'-deoxyuridine $(EdU)^+$ cells (i.e. number of EdU^+ cells corrected for hippocampal volume) in brain samples collected in early (post-hatch day (PHD) 5-7) and late (PHD 19-20) age classes during the nestling period. I found a significant treatment group by age class interaction on corrected hippocampal volume; however, I did not detect any developmental changes in corrected hippocampal volume across age classes. I also found a significant treatment group by age class interaction on the corrected number of EdU^+ cells. The corrected number of EdU^+ cells decreased across age classes in control nestlings. These results demonstrate hippocampal cell proliferation declines with age during post-hatching nestling development in a migratory songbird and this change does not affect hippocampal volume.

Second, I examined the effects of an early acute sublethal exposure to CPF during the nestling period on body mass and the same measures in the hippocampus in the same age classes. CPF exposure was confirmed by a decrease in ChE activity measured in blood samples taken the day after exposure. Moreover, I found that males had lower ChE activity than females after CPF exposure. I found a significant effect of the treatment group by age class interaction on corrected hippocampal volume. Corrected hippocampal volume was larger in CPF-dosed nestlings relative to control nestlings in the late age class, but not different in the early age class, or different across age classes in CPF-dosed nestlings. Moreover, I found a significant effect of the treatment group by age class interaction on the corrected number of EdU^+ cells. I did not find any significant difference in the corrected number of EdU^+ cells between control and CPF-dosed nestlings in the early or late age classes. However, unlike the decrease in the corrected number of EdU^+ cells across age classes I found in control nestlings, there was no decrease in the corrected number of EdU^+ cells across age classes in CPF-dosed nestlings. I did not find any effects of CPF exposure on body mass, indicating that the effects CPF had on the hippocampus were likely directly due to CPF exposure and not indirectly due to early nutritional stress. Moreover, because we noted no decrease in the corrected number of EdU^+ cells in the CPF-dosed nestlings, it is possible that CPF exposure induced compensatory cell production resulting in an increase in the corrected hippocampal volume we detected in late age class nestlings. Overall, these results demonstrate that a single exposure to CPF early in the nestling period can directly alter normal hippocampal development, through effects on hippocampal volume and cell proliferation.

Brain plasticity

As demonstrated in chapter 2, the brain is highly plastic during post-hatching nestling development in migratory starlings. Cell proliferation is a fine-scale measure of brain plasticity. Brain plasticity after hatching has been demonstrated in other birds such as zebra finch (Taeniopygia guttata) (Kim et al., 2006), domestic chicks (*Gallus gallus domesticus*) (Mezey et al., 2012) and quails (*Coturnix japonicus)* (Nikolakopoulou et al., 2006; Nkomozepi et al., 2018). However, the literature provides evidence of brain plasticity throughout life in birds (e.g. Barnea & Nottebohm, 1994; LaDage et al., 2011; Moaraf et al., 2020; Tramontin et al., 2000) and mammals (Burger et al., 2014; Migaud et al., 2011). Measures of brain plasticity, such as the number of neurons and glial cells, brain size and number of neuron synapses, can be influenced by different factors (reviewed in Kolb & Whishaw, 1998). For example, in birds, brain plasticity can be influenced by intensity of food storing (Barnea & Nottebohm, 1994; LaDage et al., 2010) and differences in social groups (Lipkind et al., 2002). Moreover, each measure can be influenced either positively or negatively by different factors (reviewed in Kolb & Gibb, 2011), such as environmental enrichment (Kempermann et al., 1997) and stress (Coe et al., 2003). The results in chapter 2 demonstrated significant brain plasticity in an altricial species during the post-hatching nestling period; however, because starlings are migratory and rely differentially on spatial cognition throughout the seasons, it is likely that further changes will occur later in life (LaDage et al., 2011).

Impacts of agricultural insecticides on wildlife

As I showed in chapter 2 of this thesis, the OP CPF can alter normal developmental changes in the hippocampus of migratory starlings. These results add to the literature that shows

that the widespread use of insecticides in the agricultural industry is of concern due to the detrimental effects that insecticides like OPs and more recently, neonicotinoids, can have on wildlife. Other studies have demonstrated numerous effects that insecticides have on wildlife. For example, diazinon exposure during PND 1-4 caused short-term hyperactivity and affected working memory in a radial arm maze test later in life in rats (Timofeeva et al., 2008). Exposure to a neonicotinoid, imidacloprid, during development or in early adulthood resulted in smaller mushroom body calycal growth and affected sucrose responses in bees (*Bombus terrestris audax*) (Smith et al., 2020). Imidacloprid caused white-crowned sparrows (*Zonotrichia leucophrys leucophrys)* to lose weight and fat, and decrease food intake and caused individuals to remain at the stopover site for more days (Eng et al., 2019). Overall, the results in chapter 2 of this thesis make an important contribution to our understanding of the effects of insecticide exposure on non-target wildlife.

In chapter 2 of this thesis, I measured the effect of insecticide exposure at large- and fine-scale in individual starlings; however, the use of insecticides can also have impacts on birds and pollinators at a much larger ecological scale. Birds provide numerous ecosystem services, including but not limited to dispersing seeds and controlling pests (reviewed in Whelan et al., 2015). Pollinators are also important for global food security and human health (M. R. Smith et al., 2015) Avian populations have been noted to be declining in size within North America (Rosenberg et al., 2019) and pesticide use has been suggested to be a major factor causing population declines (Mineau & Whiteside, 2013; reviewed in Stanton et al., 2018). Furthermore, insects have also been noted to be declining worldwide, including pollinators, with pesticides being the major cause (van der Sluijs, 2020). The population declines of birds and pollinators

due to agricultural intensification are thus not only affecting bird and pollinator health and populations, but also affecting fundamental ecological processes and human lives.

Study limitations

One limitation comes from our body mass analyses. Because brain samples are a terminal sample, sample size decreased in later age groups. The differences in sample sizes at each age group may have caused homogeneity issues when attempting to complete a repeated measures mixed model.

Another issue stemming from our all analyses is the use of age class as a factor instead of PHD. Because not all individuals were measured and samples were not collected on precisely the same post-hatch day due to logistical constraints in working with free-living nestlings, each age class has measurements and samples from nestlings over a range of 2 to 3 days. However, because the timing between each age group is relatively consistent for all nestlings, we believe the use of age class instead of PHD makes this limitation negligible.

Another methodological limitation in this study is the number of brains with missing sections, especially in the early age group, resulting from the delicate nature of brain tissue from undeveloped nestlings. Missing sections necessitated the use of numerous adjacent and non-adjacent sections in all brains and the function of "Account for missing section", meaning that our sampling scheme was not always completed with a systematic interval. To compensate for this issue, we stained extra sections for each brain and chose the best combination of sections possible. This enabled us to have a section interval close to every 20^{th} for all brains and reduced the number of adjacent/non-adjacent/missing sections we had to use. However, we achieved a

$CE_{m=1} > 0.1$ for all brains. Thus, the sections used, and the sampling parameters likely provided accurate hippocampal volume and EdU^+ cell count estimates, making this effect negligible.

Another limitation in this study is that we were unable to process middle age class brains and we had low sample sizes, especially for EdU^+ cell counts. Due to logistical constraints, we did not process any middle age class brains and we did not include any brains that had missing sections for EdU^+ cell counts. However, middle age class brains will be added for the hippocampal volume analyses, and EdU^+ cell count data for brains with missing sections missing sections data will be collected in the near future to increase sample sizes.

Future directions

Normal developmental processes can be critical to organism behaviour, cognition, and more generally, fitness. However, there is a gap in the literature examining how large- and fine-scale measures in the brain change over a very short time scale during development in brain regions critical for cognition, such as the hippocampus. Some studies on birds compare measures in the hippocampus between nestlings or juveniles and adults (e.g. Healy & Krebs, 1993; LaDage et al., 2011) or they compare changes in fine-scale measures over a longer time scale, such as weeks (Nkomozepi et al., 2018) to months (Meskenaite et al., 2016). However, to the best of my knowledge, no study has tracked the developmental process of the hippocampus over a short time scale. Furthermore, the literature specifically on migratory bird brain development is limited. Thus, the results in chapter 2 of this thesis make important contributions to the understanding of changes in hippocampal volume and cell proliferation in a migratory species over the post-hatching nestling period. However, there is a need to measure changes in other fine-scale neural measures, such as neurogenesis and gliogenesis, over a short time scale in the

hippocampus of migrants, and examine the development of other brain regions linked to migration, like the optic lobe (Vincze et al., 2015). Examining these changes will provide a fundamental understanding of brain development in migrants and provide insight into when vulnerable periods of toxicity may be, which is important for designing ecotoxicological studies.

Wildlife are particularly susceptible to exposure to a wide array of environmental contaminants. In addition to insecticides, numerous other chemicals are present in the environment, putting wildlife at risk. For example, methylmercury exposure combined with food stress during summer increased corticosterone concentrations during the migration period in song sparrows (*Melospiza melodia)* (Bottini et al., 2022). Moreover, exposure to polychlorinated biphenyls (PCBs) during the nestling period impaired migratory orientation and moulting timing during the migration period in starlings (Flahr et al., 2015). Synthetic chemicals are suggested to be a leading driver in environmental changes; however, there is a large literature gap on the effects of chemicals on the environment (Bernhardt et al., 2017). Although some literature exists examining the effects of contaminants on wildlife, most studies measuring toxicological effects of contaminants primarily use rodents under controlled laboratory conditions. Certain biological and ecological factors such as sex, age and behaviour, can influence the effects of contaminants (reviewed in Saaristo et al., 2018). Moreover, species differ in their sensitivity to certain contaminants (reviewed in Saaristo et al., 2018). Thus, making decisions based on the effects observed in controlled laboratory experiments on rodent species for wildlife is a challenge. As such, future research should examine the effects of insecticide exposure and other contaminants on numerous measures that are significant for wildlife fitness in a variety of species to ensure that we limit all potential effects of insecticides on non-target organisms. Performing

experiments, such as the one in chapter 2 of this thesis, earlier on in insecticide production can ensure a more proactive approach on wildlife and environmental protection.

Conclusions

Overall, the results in this study are important in furthering our understanding of developmental processes within migratory songbirds and how exposure to insecticides can alter normal developmental processes. This thesis had two primary objectives: I 1) aimed to determine whether there are developmental changes in the hippocampus of a migratory songbird, at a large-scale (hippocampal volume) and a fine-scale (cell proliferation), and 2) aimed to determine whether early acute sublethal chlorpyrifos disrupts developmental changes in body mass and the same hippocampal measures. Within chapter 2 of this thesis, I was able to meet both objectives. I provided evidence that there are developmental changes in the corrected number of EdU^+ cells in the age classes I measured and there are insecticide-induced effects on corrected hippocampal volume and the corrected number of EdU^+ cells. Overall, large- and fine-scale measures in the brain are powerful and sensitive tools to measure the impact of contaminants on wildlife and potentially any stressor. However, more research is needed on both developmental processes and on the effects of insecticides on wildlife. It will be important to examine other fine scale measures during development in migratory birds, such as neurogenesis and gliogenesis, which would allow us to examine the production of neurons and glial cells over development. Furthermore, it is important to continue researching the effects of insecticide exposure in non-target wildlife species, like birds, especially because pesticides are a major driving factor in avian population declines (Rosenberg et al., 2019).

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