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Effects of methylmercury and unpredictable food stress exposure on songbirds' physiology and seasonal transition

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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Abstract

Organisms regularly adjust their physiology to respond to predictable seasonal or environmental variation. However, annual cycle transitions could be disrupted by contaminants or stressors. For example, methylmercury and stress exposure can independently disrupt birds' neural and endocrine systems, energy balance, metabolism, or behaviour, all necessary for seasonal transitions. Although, the effects of combined exposure to stressors and methylmercury (MeHg), and how long they last after exposure ends, are poorly understood. The objective of my PhD was to evaluate the impact of MeHg exposure on songbirds' physiology and its potential carry-over effects on seasonal transitions. I exposed song sparrows (*Melospiza melodia*) to environmentally relevant doses of MeHg in combination, or not, with unpredictable food stress. I observed birds' physiological changes throughout two seasonal transitions: summer to fall and winter to spring. In Chapter 2, I demonstrated that MeHg is sequestered within feathers at the time of feather growth, making feathers an appropriate tool for bird monitoring under the condition that moult pattern is well characterised in the monitored species. In Chapter 3, I found that unpredictable food stress increased body condition but decreased basal metabolic rates, while MeHg exposure increased moult duration and feather mass/length ratio in fall. In Chapter 4, MeHg and stress differently affected nocturnal fall migratory activity and the combined treatment group had increased fecal corticosterone metabolites post-exposure; both measures were positively correlated. In contrast, in Chapter 5, MeHg exposure had no detectable effect on winter to spring changes in brain GnRH cells, testosterone levels or testis size. Thus, spring reproductive onset might not be affected by MeHg in birds. However, MeHg exposure did affect cloacal protuberance, fecal corticosterone metabolites and brain neurogenesis, suggesting that exposed birds' mating success could be reduced later on. Overall, my thesis main findings were: i) except for corticosterone concentrations food stress did not exacerbate the effects of MeHg exposure, and ii) effects of MeHg on moult, migratory behaviour and secondary sexual signals may be a potential cause of concern for populations. My research highlights the importance of studying contaminant effects over multiple seasons and post-exposure periods when assessing risk for wildlife.

Keywords

Songbird, seasonality, physiology, hormones, unpredictable food stress, methylmercury

Summary for Lay Audience

Organisms adjust their physiology with seasonal or habitat changes. However, contaminants or stressors may reduce how well they perform these changes. It is not well known how combined exposure to stressors and methyl-mercury affect an organism's physiology. And how long the effect last after the exposure ends remains unknown.

My PhD aimed to assess if stress exacerbates methyl-mercury effects on songbirds' physiology and their consequence on seasonal transitions. I exposed song sparrows to unpredictable food stress and/or doses of methyl-mercury. Doses of methyl-mercury were similar to that found in insects in polluted areas. Bird's stress was induced by randomly preventing them to access their food, simulating an acute food shortage in natural habitats. I observed the birds' physiological changes throughout two seasonal transitions: summer to fall and winter to spring.

I demonstrated that feathers sequester methyl-mercury during their growth, making feathers a useful tool for bird monitoring programs. Furthermore, methyl-mercury and food stress affected the birds through different mechanisms: food stress increased birds' body condition and decreased their metabolic rates, while methyl-mercury prolonged birds' feather moult and decreasing feather mass/length ratio. When combined, methyl-mercury and stress increased levels of the hormone corticosterone but only after exposure. Corticosterone was also correlated with migratory behaviour. During the winter to spring seasonal transitions, methyl-mercury exposure did not affect the bird's reproductive onset. However, methyl-mercury increased corticosterone levels, reduced the development of cloacal protuberance, and the number of new neurons in the brain. This suggests that methyl-mercury exposure could hinder bird's mating success later on.

In summary, except for corticosterone levels, food stress did not exacerbate the effect of methyl-mercury exposure. But methyl-mercury's effects on moult, migratory behaviour and other components used for mate selection could affect populations over the long-term. In conclusion, it is important to study contaminant post-exposure effects over multiple seasons if we want to better assess risk for wildlife.

Co-Authorship Statement

Some parts of Chapter 1 come from a publication *in preparation*, authored with Scott A. MacDougall-Shackleton. I contributed to conceptualization, investigation, methodology, data curation, formal analysis, visualization, writing – original draft, writing – review and editing.

A version of Chapter 2 has been published in *Science of the Total Environment* (775: 145739) with S. A. MacDougall-Shackleton, B. A. Branfireun, K. A. Hobson. For this work, I contributed to conceptualization, investigation, methodology, data curation, formal analysis, visualization, writing – original draft, writing – review & editing. This chapter is reproduced in this dissertation with permission from the publisher Elsevier in accordance with their journal policy https://www.elsevier.com/about/policies/copyright.

A version of Chapter 3 will be submitted for publication and co-authored with R. E. Whiley, B.A. Branfireun and S.A. MacDougall-Shackleton. For this work, I contributed to conceptualization, investigation, supervision, methodology, data curation, formal analysis, visualization; writing - original draft; writing - review & editing.

A version of Chapter 4 has been published in *Hormone and Behaviour* (146: 105261), and was co-authored with R. E. Whiley, B. A. Branfireun, S. A. MacDougall-Shackleton. For this work, I contributed to conceptualization, investigation, supervision, methodology, data curation, formal analysis, visualization; writing - original draft; writing - review & editing. This chapter is reproduced in this dissertation with permission from the publisher Elsevier in accordance with their journal policy https://www.elsevier.com/about/policies/copyright.

A version of Chapter 5 will be submitted for publication and co-authored with C. Henry, Y. Zhang, B. A. Branfireun S.A. MacDougall-Shackleton. For this work, I contributed to conceptualization, investigation, supervision, methodology, data curation, formal analysis, visualization; writing -original draft; writing - review & editing.

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Chapter 1

1. General introduction

In a general context of increasing urbanisation (Seto et al., 2012; Strokal et al., 2021; UN, 2015), agricultural intensification (Parson, 1999; Rudel et al., 2009) and climate change (Kaushal et al., 2014; Noyes et al., 2009; Schindler, 2001), humans and other free-living organisms become increasingly exposed to numerous contaminants and stressors (reviewed in Acevedo-Whitehouse and Duffus, 2009; Stanton et al., 2018). Because toxicants spread is associated with decreased human health (Chen et al., 2004; Clougherty and Kubzansky, 2009; Yang et al., 2021) and ecosystem quality (Hack et al., 2020; Rowe et al., 2016; Wallace et al., 1996), it is imperative to study their effects on our environment and the organisms that inhabit it. Although population declines have not always been directly attributed to increases in pollution, toxicants exposure is certainly a contributing factor that may exacerbate selection pressure on individuals (reviewed in Fleeger et al., 2003; Fuller et al., 2022; Holmstrup et al., 2010). In order to efficiently conserve species of interest and evaluate contamination risk for populations, it is important to understand factors that may influence individual performance and fitness.

Considering multiple stages of an organism's annual cycle can improve our ability to understand how seasonality and contaminant exposure may drive population change. While reproduction is a key annual cycle stage associated with population dynamics and health, other stages are also important for individual and population survival and may carry-over to affect reproductive outcomes (reviewed in Calvert et al., 2009; Harrison et al., 2011). In contaminated sites, pollution is present year-round and may affect species differently as seasons change. Indeed, seasonal change induces numerous physiological modifications of organisms that may make them more or less susceptible to exposure to pollutants. Moreover, except for some long-term studies in humans (Kinjo et al., 1993; Weiss et al., 2002; Yorifuji et al., 2018), most toxicological research focused on short term impacts during contaminant exposure and do not assess long-term multi-season effects, nor do they usually evaluate carry-over or latency effects.

In addition to pressures associated with pollution exposure and seasonal transitions, in the wild, organisms also face multiple challenging or stressful situations. The term ‘stressor’ has many definitions, but one generally accepted is that it is a cue perceived by an organism as a threat to its current well-being, homeostasis (defined as physiological or energetic stability; McEwen and Wingfield, 2010) or survival (Goldstein and Kopin, 2007; Goldstein and McEwen, 2002). Thus, a predator presence or attack or an **unpredictable** food shortage can be considered as stressors. In comparison a challenging situation is a **predictable** event that is usually energetically costly and induces a shift in the organism’s homeostatic balance. Thus, challenges occur during migration, moult or predictable changes in temperature or food availability. On their own, these stressful or challenging events could also impact organism performance on the short and long term (Jones and Ward, 2020; Kitaysky et al., 2007) and exert selection on population traits (Relyea, 2001). Multiple authors warn against the cumulative impact of contaminant exposure and stressors on population health (reviewed in Couillard et al., 2008; Gordon, 2003; Hooper et al., 2013), but more longitudinal research on cumulative effects is needed. Overall, more studies on combined exposure to contaminants and challenges/stressors are warranted.

Bird populations, and particularly migrating or insectivorous songbird species, are decreasing in North America and over the world (Møller et al., 2008; Rosenberg et al., 2019b; Stanton et al., 2018). Hence, they are important species of interest for conservation and ecotoxicological studies. The main anthropogenic causes of direct avian mortality are cats, window collision, and other kinds of collision (e.g., cars, power lines; Erickson et al., 2005; Loss et al., 2015). Indirect mortality threats to birds are habitat loss, harm caused by invasive species, pollution, habitat overexploitation and disease (in decreasing order of threat; reviewed in Wilcove and Master, 2005). Environmental pollution is a cause of species decline (Hoffman et al., 1990; Stanton et al., 2018). For example, population decline of farmland birds have been directly and indirectly linked to pesticide toxicity (Mineau and Whiteside, 2013; Stanton et al., 2018) and agricultural intensification (Donald et al., 2006; Wretenberg et al., 2006). In general, birds are among the organisms most affected by pollution (Isaksson, 2010) and songbirds can be used as sentinels of environmental contamination (Burger and Gochfeld, 2004; Egwumah et al.,

2017; Jackson et al., 2015). Indeed, terrestrial insectivorous or vermivorous bird species can have similar or slightly lower burdens of biomagnifying pollutants such as methylmercury (MeHg), as do piscivorous or carnivorous species (Ackerman et al., 2016a; Evers et al., 2005). However, outside of lethal dose exposure it is difficult to evaluate how much reduction of population sizes is caused by reduced reproductive and physiological performance due to sub-lethal effects of contaminant exposure (Fleeger et al., 2003). In particular, the threat of contaminant like MeHg to populations remains understudied (Seewagen, 2010).

My research goals are to document how environmental pollution could exacerbate stress exposure effects and seasonal transition pressures (timing and/or energetic cost of transition) for songbirds, and to identify potential post-exposure carry-over effects. For this PhD thesis I focused on methylmercury exposure due to its ubiquity (UNEP, 2013) and documented effects in birds (reviewed in Whitney and Cristol, 2017). To better explain why such a question is important to answer, I first need to describe how each factor affects birds. Below, I first provide an overview of the physiological changes associated with seasonal transitions and annual cycle stages in birds. I particularly focus on neuronal, hormonal and body condition changes across different annual cycle stages (reproduction, moult, migration, wintering) since these are the main measures in my research. Then, I explain how birds respond to stressful situations. I then discuss how contaminant toxicity is usually assessed in birds to highlight the lack of long-term studies on carry-over effects. Next, I summarise how MeHg spreads in the environment and causes physiological disruption in wildlife in general, and birds more specifically. I then expand on how MeHg may interact with food stress to influence physiological balance associated with seasons and I elaborate on potential mechanisms behind carry over-effects. I finish this introduction by detailing my thesis objectives and thesis structure. Summarizing research that examined MeHg mechanism of action is crucial for identifying and targeting future study efforts toward species and life-stages most at risk of adverse effects. By better assessing risk throughout a species' annual cycle stages and understanding the consequences of such risks, we can more accurately inform decisions on population conservation and authorised contaminants levels.

1.1. Seasonality

1.1.1. Main mechanisms of seasonal transition

Seasonal transitions are the outcome of multiple complex mechanisms that are species- and life-stage specific, and not yet fully understood. Seasons are associated with variation in photoperiod and environmental conditions, such as temperature and precipitation (Lisovski et al., 2017), variation in resource quantity or accessibility (Guillet and Crowe, 1987), and variation in risks such as predator presence (Borgmann et al., 2013; Husby and Hoset, 2018; Mappes et al., 2014), disease (Altizer et al., 2006) or parasites (Ferreira et al., 2017; Pérez-Rodríguez et al., 2015). These varying factors have a strong impact on bird species' distribution and their phenology, particularly at higher latitudes where seasonal changes are more extreme (Bradshaw and Holzapfel, 2010; Chown et al., 2004; Tellería and Pérez-Tris, 2003). For example, access to food for insectivorous or granivorous species would be reduced in winter compared spring and summer, driving them to either migrate or to store enough food reserves before winter.

Within each season and throughout their full annual cycle stages, organisms are selected to have optimal performance to cope with environmental conditions (*beneficial acclimation hypothesis*; Leroi et al., 1994). In seasonally variable environments, individuals with traits that allow them to optimally adjust to seasonal changes are predicted to have higher fitness (Lande and Arnold, 1983; McQueen et al., 2019; Schluter, 1988). Evolution has thus resulted in birds (and other organisms) expressing season-specific morphological, physiological, and behavioural traits, whose onset and duration are influenced by seasonal environmental changes and endogenous rhythms. For example, in migratory birds seasonal changes may involve transitions between multiple annual cycle stages such as reproduction, moult, fall migration, overwintering, and spring migration (Wingfield, 2008).

The transitions between seasonal or annual cycle stages require organisms to alter their metabolism, neuroendocrine systems, and immune function (reviewed in Chmura et al., 2022; Stevenson et al., 2022; Swanson, 2010). Correspondingly, hormones and other physiological parameters usually show circadian and seasonal rhythmicity in numerous

species (McGuire et al., 2010; Rani et al., 2017; Wingfield, 2018). For example, in birds, adrenal gland responses to stimuli change with seasons (Romero, 2002; Romero, 2006; Romero and Wingfield, 1998). However, shifts between annual cycles stages must occur within time constrains precise enough so that the bird's traits closely match their environment features. Hence, birds have developed mechanisms to first detect or predict the change in their environment and then adjust their physiology, morphology and behaviour accordingly (reviewed in Bradshaw and Holzapfel, 2007; Farner, 1970; Helm et al., 2017).

Seasonal transitions first require time perception and thus a clock system (reviewed in Cassone, 2014; Stevenson and Kumar, 2017). Compared to mammals, birds do not have one master-clock system but rather have multiple clock components in different areas, including the retina, the pineal gland (secreting melatonin) and the suprachiasmatic nucleus (SCN) that are connected and synchronized with each other and with the peripheral tissues' own clock mechanisms (reviewed in Cassone, 2014; Kumar et al., 2010). These clock components dictate the birds' endogenous circadian and circannual rhythms. For example, endogenous programing can induce free-run onset of reproductive physiology (Budki et al., 2012; Budki et al., 2014; Gwinner, 1989), migratory behaviour (Gwinner, 1989; Gwinner and Wiltschko, 1980; Holberton and Able, 1992), moult and body mass change (Budki et al., 2012; Holberton and Able, 1992; Piersma et al., 2008) in birds kept under constant conditions. However, these internal clock components are synchronized by external cues like photoperiod. Hence, the bird's clock-system promotes not only the circadian rhythm, but also interacts with endogenous programing and environmental cues to induce seasonal rhythm transition.

Photoperiod cues stimulate changes between annual cycle stages, serve as an endogenous rhythm entrainment mechanism and synchronize seasonal transitions with the environmental conditions (reviewed in Follett, 2015; Liddle et al., 2022; Menaker, 1971). Because variation in day-length throughout the year is one of the most reliable indicators of changes in environmental condition, birds evolved sensitivity to this cue to detect and predict forthcoming seasonal shifts (MacDougall-Shackleton et al., 2009). As such, a change in photoperiod or relative length of day-night period induces the start of neural

and physiological shifts between annual cycle stages and the coordination of organisms' physiological reactions and seasonal processes (reviewed in Dawson, 2008; Liddle et al., 2022). For example, many species that feed their young insects synchronize their reproductive season with the increasing photoperiod and temperature in spring (Bowers et al., 2016; Love et al., 2010), and with the high resource availability at this time (Hinks et al., 2015; Lundberg, 1987; Youngflesh et al., 2021). This synchronisation enhances survival rates of offspring (Brinkhof and Cave, 1997; Hipfner, 2008; Perrins, 1970).

Photoperiod is described as the initial predictive cue used by birds for seasonal transition, but other environmental factors are considered supplementary or modifying cues (reviewed in Chmura et al., 2020). Indeed, hormonal rhythms and behavioural activity are also synchronized by food availability and ingestion (Hau and Gwinner, 1992; Kumar et al., 2001; Reierth and Stokkan, 1998). Similarly, photoperiod-driven changes in migration or reproductive onset in birds are influenced by temperature (Berchtold et al., 2017; Lewis and Farner, 1973; Marra et al., 2005), food stress (Gwinner et al., 1988; Terrill, 1987), wind conditions (Conklin and Battley, 2011; Dossman et al., 2016), precipitation or water availability (Smith and McWilliams, 2014; Vleck and Priedkalns, 1985; Wingfield et al., 2012), or social cues (Gwinner et al., 2002; Helm et al., 2006; Terrill, 1987). Overall, numerous internal and external signals coordinate birds' shifts between annual cycle stages.

Now that I have described photoperiodism and seasonality in general, below I expand on the neuronal, endocrine and physiological changes between these annual cycle stages, focusing on reproduction, moult, migration and wintering.

1.1.2. Seasonal control of bird reproduction

Each onset of a seasonal shift between annual cycle stages is supported by numerous cascading events from neuroendocrinology up to morphological and behavioural modifications. For example, the onset of reproduction in birds is associated with the spring increase of day length. This photoperiodic cue directly stimulates the bird's deep brain photoreceptors and intensifies the hypothalamic-pituitary-thyroid (HPT) secretion

that in turn promote the release of gonadotropin hormone and activation of the hypothalamic-pituitary gonadal (HPG) axis (reviewed in Liddle et al., 2022; Nakane and Yoshimura, 2014). This HPG axis stimulation leads songbirds to almost completely regrow their reproductive tract and gonads (Ball, 1993; Dawson, 2015a). Gonadal growth includes the increased secretion of reproductive androgen and estrogen hormones compared to fall or winter.

Gonadal steroid hormones (e.g., testosterone, estradiol, progesterone) then stimulate several parts of the brain where processes such as neurogenesis and the number of projections between regions of the brain are intensified (reviewed in Chen et al., 2013). For example, stimulation from gonadal steroid hormones increases the size and number of neurons of song control areas and enhances myelination (Brenowitz and Lent, 2002; Güttinger et al., 1993; Stocker et al., 1994). The neuronal connections between HVC and robust nucleus of the arcopallium (RA) areas of the brain song control system are increased in association with HVC growth (Brenowitz and Lent, 2001; Meitzen et al., 2007).

These modifications in the brain leads to the onset of reproductive behaviour (reviewed in Balthazart et al., 2010). For example, androgens and neuronal changes are associated with the increase in songbirds' singing and courting behaviour (Hunt et al., 1997; Ubuka et al., 2012; Walters and Harding, 1988), or aggression for territorial defense (Silverin et al., 2004). Overall, the stimulation of HPG axis secretions in spring results in neuronal, physiological and behavioural shifts toward reproduction.

The end of reproduction is also associated with photoperiodic cues in most seasonally breeding songbirds (reviewed in Hahn et al., 2009). For example, after their reproductive peak, song sparrows (*Melospiza melodia*) enter a phase of photorefractoriness where they become less sensitive to the stimulating effects of long days and shift toward non-reproductive traits with progressive regression of gonad size and reduction of gonadal steroid secretion (Wingfield, 1993). This state of photorefractoriness terminates reproduction and gives birds time to complete moult before the onset of inclement conditions in autumn.

1.1.3. Seasonal control of moult in birds

The decrease in photoperiod and end of reproduction promotes post-nuptial moult in songbirds (Dawson, 2015b; Dixit and Sougrakpam, 2013; Wada, 1993). However, the timing of moult is flexible depending on prior reproductive activity. Free-living birds with nest failure have an earlier onset of moult and pre-migratory phenotype than reproductively successful individuals (Allard et al., 2008; Pietiainen et al., 1984; Ramos et al., 2018), while individuals with second brooding delay their moult (Mulvihill et al., 2009). Both observational studies and hormone implant experiments confirmed that, moult onset and continuation are associated with a decrease in reproductive hormones (Decuypere and Verheyen, 1986; Nolan et al., 1992; Péczely et al., 2011), glucocorticoids (Romero, 2002; Romero et al., 2005; Wingfield, 1984) and an increase in thyroid hormones (Jenni-Eiermann et al., 2002; Kuenzel, 2003; Pérez et al., 2018). Also, the stress response of birds is usually reduced during moult (Astheimer et al., 1995; Romero, 2002; Romero et al., 1998b). Overall, moult onset requires the interplay of numerous endocrine components.

This seasonal transition between breeding to a non-reproductive and moulting state is also accompanied by neuronal change. For example, in late summer song sparrows, moult is associated with a reduction in aromatase mRNA expression in some brain areas resulting in decreased aggressive behaviour compared to breeding and non-breeding seasons (Soma et al., 2003; Wacker et al., 2010). The decrease of testosterone at the end of the breeding season also results in neuronal death (Rasika et al., 1994; Thompson and Brenowitz, 2008; Thompson and Brenowitz, 2010), and the regression of song control brain regions (Thompson et al., 2007). This increased apoptosis is also coupled with increased neurogenesis in the song control system, leading song sparrows to have increased neuronal recruitment in fall than spring (Tramontin and Brenowitz, 1999) but without support from gonadal hormones, those new neurons do not persist over time (reviewed in Brenowitz and Larson, 2015).

Moult timing, duration and feather replacement sequence vary between species (reviewed in Kiat et al., 2019). Since feather replacement may hinder or eliminate a bird's flight capacity (Portugal et al., 2010; Swaddle and Witter, 1997a; Swaddle et al., 1996), moult of long distance migrant songbirds must either be completed before the start of a migratory period or be postponed during migration before finishing it on wintering sites (Kjellen, 1994; Murphy et al., 1988; Tonra and Reudink, 2018). Accordingly, long distant migrants have shorter moult duration than short-distance migrants (De La Hera et al., 2009; Kiat and Izhaki, 2016), in turn, have shorter moult than sedentary species (De La Hera et al., 2009). However, some species, usually with shorter migration routes, may finish their moult during their migration period (Niles, 1972; Norris et al., 2004a; Yuri and Rohwer, 1997). Overall, in most species, onset and duration of moult are constrained between reproduction and migration departure, supposedly to reduce overlap between energetically demanding processes (Echeverry-Galvis and Hau, 2012; Rivera et al., 1998; Svensson and Nilsson, 1997).

1.1.4. Seasonal control of migration in birds

In migrating birds, the end of summer and beginning of fall is associated with pre-migratory preparation where birds' physiology changes to prepare for high energy expenditure during flight and for habitat transition. Birds in a premigratory phase exhibit hyperphagia (Deviche, 1995; Mclandress and Raveling, 1981) with potential changes in food preference (Bairlein, 2003; Pierce et al., 2004), and body remodeling. Body remodeling occurs before and during the journey: fat stores are accumulated in large quantity (Bairlein, 2002; Johnson et al., 1989), muscles implicated in long distance movements (e.g., heart, lungs and flight muscle) increase in size and capacity while guts and other unused organs shrink (Battley et al., 2000; Bauchinger et al., 2005; Piersma and Gill, 1998). Carbohydrate, lipid and cellular metabolism are also modified (reviewed in Rani et al., 2017). Adipose, muscle tissue and blood composition change to incorporate more polyunsaturated fatty acids (Klaiman et al., 2009; McGuire et al., 2013), increased oxidative capacity (Marsh, 1981; Skrip et al., 2015; Trivedi et al., 2015) and oxygen carrying capacity (Krause et al., 2015). Modification of thermal and osmoregulation

balances (Fogden, 1972; Velten et al., 2016; Vézina et al., 2007) also occurs, presumably for acclimation to new habitat. Overall, premigratory preparation and departure decisions are controlled by an interplay between multiple hormones, endogenous and exogenous factors, many of which remain poorly understood (reviewed in Lupi et al., 2019).

Migration is associated with multiple changes in the brain. Specific areas are up- or down-regulated, influencing gene expression during premigratory preparation and migration. For example, olfactory cells proliferate to assist in orientation, memory imprinting or recalling (Agarwal et al., 2019), important for migration. Within the brain, the hippocampus, associated with spatial cognition, has larger volume and neuronal recruitment in migratory species compared to non-migratory (Barkan et al., 2014; LaDage et al., 2011; Pravosudov et al., 2006). Also, brain areas related to magnetic field detection are activated during nights of migration (cluster N; Heyers et al., 2007; Mouritsen et al., 2005; Mouritsen et al., 2016). Gene expression in the brain changes during migration, and is associated with increased neural plasticity, neuronal networks, and neural chemical and electrical signaling (Boss et al., 2016; Frias-Soler et al., 2020; Johnston et al., 2016). Neural and endocrine shifts during migration (reviewed in Stevenson and Kumar, 2017) align with behavioural adjustments such as changes from diurnal to nocturnal activity and sleep deprivation for diurnal species migrating at night (Fuchs et al., 2006; Rattenborg et al., 2004).

Migratory preparation and flight are heavily influenced by endocrine actions. For example, the thyroid hormone thyroxine (T4) appears to be a major mediator of migratory behaviour (Pathak and Chandola, 1982; Pérez et al., 2016), while corticosterone (Cort) effects are mixed for migratory preparation (Bauer and Watts, 2021). Cort has a clear role in stimulating departure and sustaining travel (Bauer and Watts, 2021); however, the hypothalamic-pituitary-adrenal (HPA) axis stress response is reduced during migration compared to breeding (Wilson et al., 2017) and is absent during migratory flight (Jenni-Eiermann et al., 2009). Together, Cort and thyroid hormones affect neurodegenerative processes (Chaker et al., 2018; Newman et al., 2010; Wada et al., 2014), muscle and cardiovascular functions, and the respiratory and immune systems (reviewed in McNabb, 2007; Sapolsky et al., 2000) that are important for migration. Melatonin also exhibits a

slight reduction in circadian cycle (Fusani and Gwinner, 2004; Gwinner and Brandstätter, 2001; Gwinner et al., 1993) and loss of circadian rhythm during migration (Trivedi et al., 2019a). This presumably allows night activity in diurnal species. For examples, the circadian melatonin cycle is reduced in seabirds active at night outside of migratory period (Wikelski et al., 2006). Among the hormones relevant to migration, ghrelin decrease food intake and fat stores, in birds while increasing migratory restlessness (Goymann et al., 2017) and mediates stopover departure (Lupi et al., 2022). This hormone modulates the energy homeostasis network along with glucocorticoids and insulin (Song et al., 2019).

While gonadal hormones have strong influences on spring migration, their role in fall migration is still not fully understood. For example, androgens (testosterone and DHT) influence hyperphagia and fattening (Deviche, 1995), while testosterone has anabolic effects on muscle mass through increasing muscle protein synthesis (Herbst and Bhasin, 2004; Mayer and Rosen, 1975). Testosterone also stimulates, but does not activate, migratory restlessness suggesting that individuals with higher testosterone had earlier migratory departure and arrival in spring (Tonra et al., 2011; Vandermeer, 2013). Similarly, in captivity, testosterone implanted birds had an earlier onset of migratory restlessness than control birds (Owen et al., 2014). Also, androgen levels were higher in individuals that overwinter closer to the breeding grounds (Lymburner et al., 2016), suggesting that androgens could influence fall migration distances and wintering location decisions. Overall, androgen effects on migratory behaviour can partially explain protandrous migration, where male migratory songbirds usually arrive on breeding grounds earlier than females (reviewed in Coppack and Pulido, 2009; Morbey and Ydenberg, 2001).

Some differences in hormone levels between migratory seasons are documented, suggesting a variation in physiological mechanisms between spring and fall migration. For example, in addition to hormones associated with the HPG axis, Cort and tyrosine hydroxylase enzyme are higher in spring migration than fall migration (Sharma et al., 2018). Similarly, in birds, the adrenal gland sensitivity to adrenocorticotropic hormone (ACTH) is lower in fall and winter (Romero, 2006; Romero and Wingfield, 1998). On the

contrary, birds' thyroid hormone tri-iodothyronine (T3) and melatonin are higher in fall than spring migration (Fusani and Gwinner, 2004; Sharma et al., 2018). Interestingly, the removal of the pineal gland perturbed orientation in fall (Schneider et al., 1994) but not spring migration (Semm et al., 1987), suggesting a role of melatonin in fall migration. Similarly, melatonin treatment reduced bird migratory restlessness in fall but not in spring (Fusani et al., 2011; Fusani et al., 2013) and food deprivation reduced melatonin and migratory restlessness in fall but not in spring (Fusani and Gwinner, 2004). Explanations for such seasonal differences are still missing but the HPG axis stimulation in spring compared to fall could be one of the causes behind it.

1.1.5. Bird overwintering physiology

To my knowledge there is little research identifying physiological or neurological characteristics specific of fall migration termination and the overwintering period in wild birds. Typically, hormone levels or gene expression of hormones associated with reproductive onset and gonadal activity are low in the blood or brain in wintering birds (Jalabert et al., 2021; Trivedi et al., 2019b). Similarly, Cort levels in blood and brain ventral tegmental area and central grey area decrease in winter compared to breeding (Jalabert et al., 2021). The cytosolic corticosteroid receptor is also lower in the non-breeding season while corticosteroid-binding globulin is lower during moult than winter that is still lower than breeding season (Breuner and Orchinik, 2001). This could then affect the transport of Cort in circulation (Breuner and Orchinik, 2002; Charlier et al., 2009) and suggests that birds may not need to, or may not have the capacity to, have a strong stress response during winter when facing unpredicted conditions. Overall, wintering ground territory quality and seasonal schedules can impact the next year's annual cycle stages (Fayet et al., 2016). However, the wintering period is usually seen as a 'resting' period giving birds the opportunity to 'reset' their physiological balance and seasonal schedule (Briedis et al., 2018; Gow et al., 2019; Senner et al., 2014).

1.2. Stressors and the stress response

In every season, free living birds face multiples stressors such as inclement weather (Krause et al., 2016; Wingfield, 1985a; Wingfield, 1985b) or food unpredictability (Doody et al., 2008; Jenni-Eiermann et al., 2008) that can affect their physiology (Boyer and MacDougall-Shackleton, 2020; Fokidis et al., 2012; Schmidt et al., 2015). Similarly, predator cues (visual, auditory, olfactory) may also change bird territory distribution (Burgas et al., 2021; Forsman et al., 2013; Vernouillet et al., 2020), negatively impact offspring number or body condition, increase adult and nestling glucocorticoid hormone secretion and affect their physiological balance (Clinchy et al., 2004; Grade et al., 2021). In suboptimal habitats, individuals may have to spend more energy to meet their biological requirements and those of their offspring (Godfrey, 2003; Hinsley et al., 2008; Stauss et al., 2005). For example, in poor-quality habitat, birds could exhibit a lower food provisioning rate due to longer foraging trips (D'Astous and Villard, 2012; Stauss et al., 2005; Tremblay et al., 2005). Hence, mounting an appropriate stress response to stressful or challenging situation without losing large energy stores or performance is a crucial trait for survival and reproduction.

When facing a stressor, birds mount a stress response that increases their chance of survival. The physiological stress response arises in two steps (reviewed in Godoy et al., 2018; Ulrich-Lai and Herman, 2009). The first step occurs within seconds of the stressor encounter and is associated with the stimulation of the parasympathetic nervous system (e.g., release of epinephrine and norepinephrine; Siegel, 1980; Wurtman, 2002). This step increases heart rate (Carravieri et al., 2016) and initiates a fight or flight response (reviewed in Goligorsky, 2001; McCarty, 2016; Romero and Gormally, 2019), that helps the bird escape the stressor and survive the immediate threat. The second response consists in an alteration of the bird's hormones levels, physiology and metabolism. This second step takes slightly longer to take place compared to the first one but has effects that can last from hours up to months in cases of chronic stress (reviewed in Sapolsky et al., 2000; Siegel, 1980). For example, in birds, the stimulation of the HPA axis takes 2 minutes before glucocorticoid levels increases are detected (Romero and Reed, 2005), and glucocorticoid levels reach a peak at 15–45 min (Astheimer et al., 1995; Lumeij et

al., 1987; Romero et al., 2006), before returning to baseline levels after 60–90 min or more of initial stressor exposure (Le Maho et al., 1992; Rich and Romero, 2005). The level of circulating Cort is a good marker of short-term stress in birds since more stressful events induce higher levels of glucocorticoids (Kitaysky et al., 2001; Romero, 2002). Along with glucocorticoid increases, acute stress physiological responses include rapid energy resource reallocation, improved immune function, inhibited digestion, increased respiration, and behaviour modifications (reviewed in Johnstone et al., 2012; Sapolsky et al., 2000).

Overall, the stress response functions and HPA axis stimulation is a beneficial adaptive response to adverse conditions since it restores an organism to homeostasis after a stressful event and promotes higher survival (Blas et al., 2007; Brown et al., 2005; Patterson et al., 2014). Glucocorticoids also have effects on learning and memory (Jones et al., 2016; Kriengwatana et al., 2015; Pravosudov, 2003) that promote more appropriate behavioural responses during the organism's next encounter with the stressor (reviewed in Pravosudov, 2007; Trollope et al., 2012). Similarly, glucocorticoids mediate physiological processes involved in the organism's energy metabolism (Astheimer et al., 1992; Fokidis et al., 2012; Jimeno et al., 2017) and hence play an important role in energy balance under stressful and challenging conditions. Stress exposure usually redirects energy allocation from long-term processes, such as growth and reproduction, to short-term survival.

Prolonged exposure to a stressor and chronic activation of the HPA axis, however, can have long-term negative effects, detrimental to the fitness of individuals (Dickens et al., 2009; Wingfield et al., 1997). For example, chronically stressed animals may have increased oxidative stress and tissue damage (Costantini et al., 2011), reduced immunity (Buchanan, 2000; Cheville, 1979; Martin, 2009; Raberg et al., 1998) and reduced neurogenesis (Robertson et al., 2017). Overall, stressor(s) presence could have a strong negative effect on individuals' reproductive success (Cyr and Michael Romero, 2007) and survival (Acevedo-Whitehouse and Duffus, 2009; Kilgas et al., 2006) that will further impact populations. Hence, it is an important factor to account and study if we want to better preserve species of interest.

From a more conceptual point of view, stress and particularly chronic stress are often associated with energetic cost. The concept of allostasis (processes of maintaining homeostatic balance through change; McEwen, 1998; McEwen and Wingfield, 2003; Wingfield, 2005) and the reactive scope model (Romero et al., 2009) provide frameworks accounting for the effects of different stressors on the energetic load of organisms and on the range of physiological mediator response (predictive homeostasis, reactive homeostasis, homeostatic overload, homeostatic failure). Both models differentiate between predictable and unpredictable stressors in the environment, but the latter is associated with the concept of wear and tear (Romero et al., 2009) where maintaining a specific physiological system or mediators induce cost. Thus, the ability of the organism to cope with the stressor decreases with time. Hence, stressors are viewed as a continuum that can accumulate over time and/or have long-term effects. Overall, the allostasis and reactive scope models work with predictable seasonal changes in the endocrine function and account for the cumulative energy costs associated with seasonal changes (allostatic load; McEwen and Stellar, 1993).

Finally, it is important to quantify multiple hormones when assessing stress effects and seasonal transitions. While the main endocrine axis affected by stress is the HPA axis, the HPA, HPG and HPT axes can interact and influence each other's actions (reviewed in Castañeda Cortés et al., 2014; Tsutsui and Ubuka, 2018). Notably, stress exposure can decrease GnRH and gonadal secretions to negatively affect reproductive traits (reviewed in Davies and Deviche, 2014; Wingfield and Ramenofsky, 2011; Wingfield and Sapolsky, 2003). HPA axis stimulation also increases HPT axis secretion (Geris et al., 1996; Meeuwis et al., 1989). Similarly, HPT secretions promote HPA function (reviewed in Carsia et al., 1985; Debonne et al., 2008; Watanabe et al., 2016) and HPG activation (Duarte-Guterman et al., 2014; Tsutsui and Ubuka, 2018; Vasudevan et al., 2002) and effects, although knowledge is scarce in birds. Hence, thyroid disruption could affect the stress response of organisms and their reproductive readiness. Overall, the interplay between multiple hypothalamic-pituitary axes within each season determines the bird's short- and long-term effects of stress.

1.3. Some generalities on avian toxicity tests

In theory, before a new chemical compound is approved and released on the market, it must first be tested *in vitro* and then on several animal species including mice or rats for human health concerns, and micro-organisms and invertebrates to test bioavailability and bioaccumulation in the soil or aquatic habitats (Anderson et al., 2004; Hooper and Anderson, 2009; USEPA, 1994). The animal testing may encompass vertebrates such as birds either if the contaminant is environmentally persistent, if there is evidence of biomagnification, if prior testing in mammals show adverse effects on reproduction or if birds or poultry may be directly exposed to the compound (Bennett and Ganio, 1991; Hooper and Anderson, 2009). The main guidelines and current requirements for avian testing are set by several organizations (reviewed in Hill and Hoffman, 1984b; Hill et al., 2014; Valverde-Garcia et al., 2018) such as the Organisation for Economic Co-operation and Development (OECD, 1984a; OECD, 1996; OECD, 2018) and United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (USEPA, 2012a) and the European Food Safety Authority (EFSA, 2009).

The main toxicity tests done in birds generally focus on survival and reproductive performance. Also, they rarely include a post-exposure period to assess for potential carry-over or latency effects on these endpoints, with the exception of multiple-generation tests. Indeed, the main suggested avian toxicity tests (OECD, 1996) include:

- An avian acute oral toxicity testing (OCSPP Guideline # 850.2100; OECD, 2016): the tests incorporate a single oral dose exposure with at least 3 different concentrations tested side by side: low dose, an estimated 50 % lethal dose (LD_{50}), and an approximate lethal dose, and a post-exposure observation period of 14 days.
- An short term avian dietary toxicity test (OCSPP # 850.2200): with exposure duration varying from 5 days exposure with at least 3 day post-exposure (OECD, 1984b; USEPA, 2012b), to 21 days plus 7 days of post-exposure observations (OECD, 1996). This test aims to identify a 50% lethal concentration exposure (LC_{50}).
- An avian reproduction test (OCSPP # 850.2300): consisting in an exposure phase of six-eight weeks long, a post-exposure photostimulation phase of two-four weeks and a

post-exposure laying phase of eight-ten weeks (USEPA, 2012a). The main endpoint of this test is to determine the No Observed Adverse Effect Concentration (NOAEC) and assess exposure effects over one or two generation (OECD TG 206 protocol; OECD, 2018).

- Other tests on avian species include the 'single-dose embryotoxicity test' and a 'subchronic dietary toxicity test' (4-9 weeks exposure; reviewed in Hill and Hoffman, 1984) and other tests for broader purposes such as non-target organism assessment: the Avian Oral, Tier 1 (OCSPP # 885.4050), the Avian inhalation test, tier1 (OCSPP # 885.4100), the Avian Two-Generation Toxicity Test, tier 2 (OCSPP # 890.2100; USEPA, 2015), avian chronic pathogenicity and reproduction test, tier 3 (OCSPP # 885.4600), or the residue chemistry test guidelines (minimum exposure of 28 days, OCSPP Guideline # 860.1480; USEPA, 1996). Tiers represent the priority of chemical needed for assessment, with tier 1 for chemicals with highest priority for environmental assessment on national or regional scale, and tier 3 for chemical compounds of low to no-priority for monitoring due to lack of effect in human and wildlife (Olsen et al., 2013).

In general, the endpoints in the tests reviewed above are typically related to adult health (e.g., body weight, food consumption, overall health, % survival to exposure dose) and reproduction (e.g., egg production, embryo viability and survival, hatchability, survival of offspring, effects on the eggshell, and the body weight of offspring; Valverde-Garcia et al., 2018). While reproductive outcomes and survival are the endpoints most relevant on a population dynamic, ecological and conservation point of view, the current avian tests are, in my opinion, very limited in addressing non-lethal endpoints or the variability in toxicological risks associated with different seasons. For example, the current reproductive endpoints do not assess the ability of an individual to take care of its offsprings (parental care behaviour).

Furthermore, the recommended species for these tests may not offer a good representation of wild species. Indeed, the main tested species are typically Japanese quail (*Coturnix coturnix japonica*), northern Bobwhite (*Colinus virginianus*) and Mallard duck (*Anas platyrhynchos*; USEPA, 2012a; Valverde-Garcia et al., 2018). Additional species

suggested are pigeon (*Columba livia*), ring-necked pheasant (*Phasianus colchicus*) and red-legged partridge (*Alectoris rufa*; USEPA, 2012b). These species are easy to breed and raise in captivity in large groups. However, they may have different physiological requirements and toxicity resistance than other wild birds (Evers, 2018; Fuchsman et al., 2017; Heinz et al., 2009; Zhang et al., 2013). Also, except for the pigeon they all have precocial chicks and are largely habituated to human presence so may not often need to mount an appropriate stress response. Hence, exposure effects on these species may not be representative of wild species that produce altricial young, such as songbirds, that also have to face multiple challenges and stressors from their natural environment. Although the use of species such as zebra finches (*Taeniopygia guttata*), American robins (*Turdus migratorius*), barn owls (*Tyto alba*), and other have also been suggested (OECD, 1996; Sample et al., 1996), as well as additional measures or endpoints (e.g., onset of egg laying, parental behaviour and social interactions) such testing protocols have not yet been approved.

1.4. Methylmercury

1.4.1. MeHg cycling in the environment

Mercury is a global pollutant and potent neurotoxin produced by both natural and anthropogenic processes such as fires, volcanoes, artisanal and small-scale gold production, coal combustion and production of non-ferrous metals (reviewed in Obrist et al., 2018; Pirrone et al., 2010; UNEP, 2013). Current environmental mercury levels are due to a combination of new inputs, continued re-emission and deposition, and the persistence of mercury in ecosystems that favour re-emission (Driscoll et al., 2013; Obrist et al., 2018; Seigneur et al., 2004).

Mercury’s ubiquity stems from its capacity to cycle through air and water compartments as well as biota. Mercury cycling into the environment has been previously reviewed (Bishop et al., 2020; Driscoll et al., 2013; Obrist et al., 2018). In its gaseous form mercury is estimated to persist between 6 months to 2 years in the atmosphere (Lindqvist and Rodhe, 1985; Slemr et al., 1985; Strode et al., 2008) and travel long distances before its

deposition in ecosystems (Durnford et al., 2010; Schroeder and Munthe, 1998; Strode et al., 2008). In contrast, the average residence time of mercury in ocean water is between 20 and 30 years (Gworek et al., 2016). Deposition occurs through wet precipitation as snow, rain, cloud and fog, or as dry deposition of particulate matter, or via other processes such as stomatal leaf uptake in plants and water surface exchange of reactive gaseous mercury. Except for particulate matter, deposited mercury normally takes the form of the more soluble inorganic mercury (divalent Hg^{2+}; reviewed in Driscoll et al., 2013).

Once deposited, conversion of inorganic mercury into bioavailable methylmercury (MeHg) occurs (Jensen and Jernelöv, 1969; Weber, 1993; Wood et al., 1968), primarily from the actions of iron- and sulfur-reducing bacteria under anaerobic conditions (Boyd et al., 2017; Merritt and Amirbahman, 2009) such as in aquatic habitats, floodplain soil and some forest soil (Bravo and Cosio, 2020; Gerson et al., 2017; Regnell and Watras, 2019). This transformation is facilitated by low pH, high dissolved organic matter, and high moisture content of soil (Merritt and Amirbahman, 2009; Paranjape and Hall, 2017; Ullrich et al., 2001). MeHg will then enter the food chain via primary producers or invertebrate incorporation and then bioaccumulates and biomagnifies (reviewed in Mahbub et al., 2017; Munthe et al., 2007), resulting in concentrations of concern at upper trophic levels (reviewed in Ackerman et al., 2016a; Chételat et al., 2020; Lehnherr, 2014).

Human activities over the past 160 years (1850-2010 CE) have resulted in a 2 to 4-fold increase in atmospheric mercury (Cooke et al., 2020; Fitzgerald et al., 2005; Streets et al., 2017). The feathers of several bird species showed an increase in MeHg content starting as early as 1900s in some species (Monteiro and Furness, 1997) or in the 1940s to 1950s in other studies, likely due to MeHg compounds being used as seed dressing (Ackefors, 1971; Berg et al., 1966). Overall, mercury emissions and deposition have been decreasing since the 1970s (see Balogh et al., 1999), thanks to increased awareness (WHO, 1976; WHO, 1989) and regulations. For example, regulation such as the US Clean Air Act of 1963, the National Environmental Policy Act in 1969, the Toxic Substances Control Act in 1976, and the Clean Water Act in 1977 helped reducing mercury emission (Balogh et al., 1999; Fairbrother, 2009). However, with increasing greenhouse gas emissions,

mercury present in the environment is predicted to increase, in association with ice melting and releasing of MeHg deposited in the past (Schaefer et al., 2020). Similarly, some studies predict increasing methylation processes and environmental levels of methylmercury due to future climate change (Jonsson et al., 2017; Krabbenhoft and Sunderland, 2013).

1.4.2. MeHg uptake and tissue compartmentalization

MeHg is a concerning contaminant due to its harmful effects on humans and wildlife (Chan et al., 2003; Dietz et al., 2013; Scheuhammer et al., 2007). In terrestrial animals MeHg exposure mainly occurs through the diet. MeHg is considered the most harmful form of mercury due to its capacity to pass the gastrointestinal barrier, with an absorption rate varying between 12-79 % in humans (Bradley et al., 2017), and especially due to its capacity to cross the blood-brain barrier (Aschner and Aschner, 1990; Yin et al., 2008) and act as a potent neurotoxin. MeHg also has a long half-life, ranging from approximately 20 to 30 days in different tissue compartments in humans (reviewed in Rand and Caito, 2019). It is thus important to identify how MeHg compartmentalises in organisms and to characterise its mode of action in order to better estimate the risk of adverse effects for individuals and populations according to levels measured in diet or tissues.

Once ingested, mercury can be found in multiple tissues. Most studies measured total mercury (THg) representing concentration of the two main chemical forms within tissues: the inorganic mercury (IHg; regrouping both the elemental and ionic forms) and MeHg (methylated with a single methyl-group). In general, the concentration of THg in avian organs follow the gradient of concentration of feather > liver > kidney > muscle > blood > brain (Dias dos Santos et al., 2021; Kim et al., 1996; Mallory et al., 2018). However, some studies have found higher concentrations in kidney than in liver (Eagles-Smith et al., 2008; Mikoni et al., 2017; Szumiło-Pilarska et al., 2016), and others have found higher concentrations in blood than muscle (Raygoza-Viera et al., 2013; Spalding et al., 2000a; Szumiło-Pilarska et al., 2016) or higher concentrations in brain than muscle

(Leonzio et al., 1986a; Leonzio et al., 1986b). Some publications also found higher THg levels in liver than in feathers (Evers et al., 2005; Hribšek et al., 2017; Kim et al., 1996). However, this is probably due to either species difference in physiology, differences in MeHg chemical form in diet or study experimental design.

Ingested MeHg accumulation and compartmentalisation within tissues depends on several factors. First, MeHg has binding affinity to selenol (selenohydryl molecules: selenoprotein P, GPx and TrxR) and, to a lower extent, thiol (sulfhydryl molecules: hemoglobin, albumin, GSH, and Cys) (Spiller, 2018; Nogara et al., 2019). MeHg also binds to keratin elements found in feathers (Crewther et al., 1965), claws (Low et al., 2020) and beak (de Medeiros Costa et al., 2021). MeHg is also fat-soluble (Halbach, 1990), thus tissue concentrations vary depending on fat composition (Aazami and KianiMehr, 2018). Individual genetic characteristics can also influence MeHg transport and compartmentalisation in tissue (reviewed in Gundacker et al., 2010), but this has not been well documented in birds. In experimental studies, tissue compartmentalisation may vary as a result the chemical form (e.g., complexed with chloride, MeHgCl, or cysteine, MeHgCys) of MeHg exposure (Roos et al., 2010) although not always (Glover et al., 2009; Varian-Ramos et al., 2017) and by the type of exposure (air, injection, diet, water contamination; Mori et al., 2012). The duration of exposure may also play a role, since longer exposures would usually mean higher accumulation levels and, in adult birds, demethylation processes only start after a threshold of 8.51 $\mu g.g^{-1}$ THg in liver (Eagles-Smith et al., 2009). The effect of exposure duration on tissue compartmentalisation has not been well documented.

Finally, MeHg accumulation in tissues also depends on the individual coping mechanisms such as demethylation (Eagles-Smith et al., 2009) or depuration such as feather sequestration (Honda et al., 1986). Constant low depuration processes in exposed birds were suggested due to low levels of THg detected in feces (Costa et al., 2012; Lewis and Furness, 1991; Spalding et al., 2000a) and preen oil gland (Frank et al., 1983; Leonzio et al., 1986a; Saeki et al., 2000). Such coping mechanisms can have a genetic basis, associated for example with the capacity to produce antioxidant enzymes and or metallothionein proteins (Branco et al., 2017; Lucia et al., 2012). But they may also

depend on the energy balance of the individual (e.g., depuration cost and energetic trade-off; Lucia et al., 2012) or on its food source where diet containing selenium or some vitamins may help alleviate MeHg damages (Chapman and Chan, 2000; Kling and Soares, 1982; Spiller, 2018). However, to my knowledge, such traits are not incorporated into the identification of species most at risk of detrimental exposure effects.

1.4.3. MeHg toxicity levels in birds

Several publications have established exposure thresholds in birds, to both assess acceptable levels of environmental contamination and to evaluate species' risks of deleterious effects of exposure to MeHg. Mercury exposure thresholds affecting reproduction are one of the most evaluated. For example, for embryo-toxicity, the egg median lethal concentration (LC_{50}) varies from 0.25-1 $\mu g/g^{-1}$ MeHg for species with medium sensitivity (Heinz et al., 2009). And for adults reproductive-deficiency, the effective concentration associated with a 20% reduction in reproductive success (EC_{20}) was determined at 1.2 $mg.kg^{-1}$ wet weight (w.w.) in blood and 3.4 $mg.kg^{-1}$ fresh weight (f.w.) in body feathers of free-living insectivorous birds (Jackson et al., 2011a). In a literature review, an EC_{20} reproductive threshold range was estimated between 2.1-4.2 $mg.kg^{-1}$ w.w. in blood of small and medium birds (Fuchsman et al., 2017). In a larger review, the authors collected all published avian toxicity thresholds for various impairments, life-stages and tissues, into a single global blood-equivalent toxicity benchmark (Ackerman et al., 2016a). They determined that the lowest documented effects occurred at a blood-equivalent THg concentration of 0.2 $mg.kg^{-1}$ w.w., and that numerous effects on health, physiology, behaviour or reproduction occurs within an exposure range of 1.0 to 3.0 $mg.kg^{-1}$ w.w. They also estimated that complete reproductive failure occurs at 4.0 $mg.kg^{-1}$ and adult mortality at 8.5 $mg.kg^{-1}$ w.w. blood-equivalent concentration. Finally, the authors estimated that 28% of birds sampled across western North America had blood-equivalent mercury concentration exceeding 1.0 $mg.kg^{-1}$ w.w. (moderate risk) and 4% exceed 4.0 $mg.kg^{-1}$ w.w. (severe risk) respectively (Ackerman et al., 2016a). Hence, an important number of birds are impacted by sub-lethal effects of MeHg exposure.

No neurotoxicity thresholds have been established for avian species. In human, the lowest-observed-adverse-effect level (LOAEL) leading to 5% risk of neurological damage in adults was set at 200 $\mu g.L^{-1}$ in blood and 50 $mg.kg^{-1}$ d.w. in adult hair (Clarkson and Magos, 2006; WHO, 1990) and a threshold of 10-20 $mg.kg^{-1}$ d.w. in maternal hair would result in 5% risk of neurological disorder in the fetus (WHO, 1990), equivalent to 50 $\mu g.L^{-1}$ w.w. in blood (Piotrowski and Inskip, 1981). The human neurotoxic threshold level of 10 $mg.kg^{-1}$ in the hair is also used for the mammal threshold in fur (Nam et al., 2012). Also, a LOAEL for neurotoxicity due to contaminated food consumption was estimated at 180 $\mu g.kg^{-1}$ of body weight/day (Mahaffey et al., 1997). In birds, a similar chronic LOAEL of 64 $\mu g.kg^{-1}$ of body weight/day was designated for adverse effects on reproduction and behaviour at (Mahaffey et al., 1997), and a general toxicity level encompassing neurological effect was proposed at concentration of 5 $mg.kg^{-1}$ THg in liver (Zillioux et al., 1993). However, clear neurotoxic thresholds have yet to be established for birds.

1.4.4. MeHg neurotoxic and physiological effects in birds

The adverse outcome pathway (AOP) framework has been developed in recent decades (Ankley et al., 2010) to assemble the sequential toxicity mechanisms of organisms exposed to a chemical substance through linked key cascading events at different levels of biological organisation (OECD, 2017). These events start from a molecular initiating interaction with a toxic substance (e.g., MeHg binding to seleno-proteins) producing key molecular, cellular, and organ tissue step responses (e.g., NMDA receptors neurotoxicity), up to adverse outcomes at individual or population levels (e.g., impaired cognition and reduced survival under MeHg exposure). Some AOPs exist for MeHg exposure (AOP-Wiki, 2022; Basu, 2015; Hooper et al., 2013; Karri et al., 2016; Pistollato et al., 2020).

The first steps of MeHg AOP inducing neurotoxicity and physiological disruption are relatively well documented in mammals (Figure 1.1). Across organisms and tissues, the common first step appears to be MeHg binding to selenol or thiol elements that results in

the blocking of amino acids, enzymes and proteins containing those elements, causing their inactivation and affecting their function (e.g. protein catalyse, transport or binding) and leading to dysregulation (AOP-Wiki, 2022; Oliveira et al., 2017; Spiller, 2018; Ynalvez et al., 2016), not fully characterised yet. For example, the most well documented effect is the blocking of antioxidant enzymes and further induction of oxidative stress (reviewed in Farina and Aschner, 2019) and cellular metabolic pathways disruption (reviewed in Aschner et al., 2007; Heimfarth et al., 2018; Novo et al., 2021). Within the brain such impacts can lead to neurotransmission alteration, and alteration in neurogenesis and cell death (reviewed in Castoldi et al., 2001; Johansson et al., 2007; Raposo et al., 2020). MeHg can also induce dysregulation of multiple cellular pathways associated with DNA damage, and epigenetic or post-translational modifications (reviewed in Crespo-López et al., 2009; Ke et al., 2019; Khan et al., 2019). Ultimately these neurotoxic effects (Figure 1.1) could lead to behavioural modification, embryotoxicity and reduced adult reproduction.

Figure 1.1: Main neurotoxic modes of action and adverse outcome pathway of MeHg exposure. The connection between oxidative stress induction and cellular pathway disruption is not indicated for readability. Note that other MeHg modes of action occurring outside the brain and individual level of neurotoxicity (e.g., embryotoxicity, behaviour and reproductive outcome impairment) are not highlighted here. Figure inspired from Sasaki et al., (2020) and AOP-Wiki, (2022).

I reviewed here the impact of MeHg in avian neurology. The neurological measures most consistently affected by MeHg exposure in birds are markers of oxidative stress. NMDA neurotransmitters, acetylcholinesterase, and Purkinje cells also seem sensitive to some extent (Table 1.1). MeHg exposure has the potential to affect most brain neurotransmitter system pathways, but more studies are needed for validation in birds. However, results from experiments are not always consistent with field studies and not useful to construct AOPs in birds. Overall, I encourage more research on terrestrial insectivorous species such as songbirds, especially for immature fledging age and non-reproductive adult life stage, and call attention to the need for studies of latency and carry-over effects across annual cycle stages, after birds stop ingesting MeHg. Overall, more research is needed to precisely assess short- and long-term impact of MeHg on avian populations.

Table 1.1: Summary of the main observed effects of MeHg on the avian brain. Table created from primary research articles measuring neurological or physiological effects of MeHg exposure on avian brains, resulting in 47 primary scientific articles selected. From those, I only kept the measures studied in 3 or more of the publications, excluding observational data without statistical analysis. The 'Number of studies.' column specifies the number of articles that found at least one significant effect of MeHg exposure on the measure, over the total number of publications that quantified this measure. In the 'Direction' column, the arrows indicated if the measure increased or decreased in exposed individuals compared to control individuals. The term 'species dependent' is used when the study observed different directional change in the measure depending on the species tested. The term 'life stage dependent' indicates a change in the direction of effect on the measure in exposed individual depending on their age or annual cycle stages.

Dependent measure / Reference	*Number of studies* / Direction	Species
brain size or mass	*2/7*	
Hill and Soares, 1984	-	Japanese quail, *Coturnix japonica*
Custer et al., 1997	-	Great blue heron, *Ardea herodias*
Loerzel et al., 1999	-	Double-crested cormorant, *Phalacrocorax auritus*
Henny et al., 2002	**species dependent**	Double-crested cormorant, *Phalacrocorax auritus;* Snowy egret, *Egretta thula;* Black-crowned night-heron, *Nycticorax nycticorax*
Hoffman et al., 2009	-	Snowy egret, *Egretta thula*
Kenow et al., 2011	↘	Common loon, *Gavia immer*
Yu et al., 2017	-	Zebra finch, *Taeniopygia guttata*
Purkinje cells count	*2/3*	
Carvalho et al., 2008	↘	Chicken, *Gallus domesticus*
Rutkiewicz et al., 2013	-	Herring gulls, *Larus argentatus*
Scoville et al., 2020	↗	Zebra finch, *Taeniopygia guttata*
Adenosine triphosphate (ATP) content	*1/3*	
Hoffman and Heinz, 1998	-	mallard duck, *Anas platyrhynchos*

Henny et al., 2002		↘	multiple
Kenow et al., 2008		-	Common loon, Gavia immer
NMDA & glutamate receptors	*3/7*		
Scheuhammer et al., 2008		↘	Common loon, *Gavia immer*; Bald eagle, *Haliaeetus leucocephalus*
Rutkiewicz et al., 2010		↘	Herring gulls, *Larus argentatus*
Hamilton et al., 2011		-	Common loon, *Gavia immer*
Braune et al., 2012		-	Thick-billed murre, *Uria lomvia*; Arctic tern, *Sterna paradisaea*
Rutkiewicz et al., 2013		↗	white leghorn chicken, *Gallus gallus domesticus*; Japanese quail, *Coturnix japonica*
Van den Brink et al., 2018		-	Barnacle goose, *Branta leucopsis*
Muscarinic acetylcholine receptors (mACh)	*1/4*		
Scheuhammer et al., 2008		↗	multiple
Rutkiewicz et al., 2010		-	Herring gulls, *Larus argentatus*
Hamilton et al., 2011		-	Common loon, *Gavia immer*
Braune et al., 2012		-	multiple
Acetylcholinesterase (AChE) enzyme activity	*3/7*		
Hill & Soares, 1984		↘	Japanese quail, *Coturnix japonica*
Hoffman and Heinz, 1998		-	mallard duck, *Anas platyrhynchos*
Henny et al., 2002		-	multiple
Hoffman et al., 2005		↗	Great egret, *Ardea alba*
Ji et al., 2005		-	domestic ducks, shaoxing duck
Kenow et al., 2008		-	Common loon, Gavia immer
Hoffman et al., 2009		↗	Snowy egret, *Egretta thula*
Cholinesterase (ChE) enzyme activity	1/5		
Dieter and Ludke, 1975		-	Japanese quail, *Coturnix coturnix japonica*
Dieter & Ludke 1978;		-	Japanese quail, *Coturnix coturnix japonica*
Wolfe and Norman, 1998		-	Great blue heron, *Ardea herodias*
Scheuhammer et al., 2008		↘	multiple
Hamilton et al., 2011		-	Common loon, *Gavia immer*

Glucose-6-phosphate dehydrogenase (G-6-PDH)	*3/6*		
Hoffman and Heinz, 1998		**↘**	mallard duck, *Anas platyrhynchos*
Henny et al., 2002		**↗**	multiple
Hoffman et al., 2005		**-**	Great egret, *Ardea alba*
Kenow et al., 2008		**-**	Common loon, Gavia immer
Hoffman et al., 2009		**-**	Snowy egret, *Egretta thula*
Hoffman et al., 2011		**life stage dependent (↗ mainly)**	Forster's tern, *Sterna forsteri*; Caspian tern, *Hydroprogne caspia*
Glutathione oxidized (GSSG)	*2/5*		
Henny et al., 2002		**-**	multiple
Hoffman et al., 2005		**-**	Great egret, *Ardea alba*
Kenow et al., 2008		**↗**	Common loon, Gavia immer
Hoffman et al., 2009		**↘**	Snowy egret, *Egretta thula*
Hoffman et al., 2011		**-**	multiple
Glutathione reductase (GSSG-Red)	*5/6*		
Henny et al., 2002		**↘**	multiple
Hoffman et al., 2005		**-**	Great egret, *Ardea alba*
Kenow et al., 2008		**↗**	Common loon, Gavia immer
Carvalho et al., 2008		**↗**	Chicken, *Gallus domesticus*
Hoffman et al., 2009		**↗**	Snowy egret, *Egretta thula*
Hoffman et al., 2011		**↘**	multiple
Glutathione reduced (GSH)	*4/8*		
Hoffman and Heinz, 1998		**-**	mallard duck, *Anas platyrhynchos*
Henny et al., 2002		**↘**	multiple
Hoffman et al., 2005		**↗**	Great egret, *Ardea alba*
Ji et al., 2006		**↗**	domestic ducks, shaoxing duck
Carvalho et al., 2008		**↗**	Chicken, *Gallus domesticus*
Kenow et al., 2008		**-**	Common loon, Gavia immer
Glutathione S-transferase (GSH-S-T)	*1/4*		
Henny et al., 2002		**-**	multiple

Hoffman et al., 2005		-	Great egret, *Ardea alba*
Kenow et al., 2008		-	Common loon, Gavia immer
Hoffman et al., 2009		↘	Snowy egret, *Egretta thula*
Glutathione peroxidase (GPx)	*3/3*		
Hoffman et al., 2005		↘	Great egret, *Ardea alba*
Carvalho et al., 2008		↗	Chicken, *Gallus domesticus*
Cheng et al., 2013		↗	ducks (name not specified)
Selenium-dependent glutathione peroxidase (S-GPx)	*2/4*		
Henny et al., 2002		-	multiple
Ji et al., 2006		↗	domestic ducks, shaoxing duck
Kenow et al., 2008		↘	Common loon, Gavia immer
Hoffman et al., 2009		-	Snowy egret, *Egretta thula*
Total glutathione peroxidase (T-GPx)	*2/3*		
Henny et al., 2002		**species dependent**	multiple
Kenow et al., 2008		↘	Common loon, Gavia immer
Hoffman et al., 2009		-	Snowy egret, *Egretta thula*
Glutathione ratio oxidized to reduced (GSSG:GSH)	*3/5*		
Henny et al., 2002		↘	multiple
Hoffman et al., 2005		-	Great egret, *Ardea alba*
Kenow et al., 2008		↗	Common loon, Gavia immer
Hoffman et al., 2009		↘	Snowy egret, *Egretta thula*
Hoffman et al., 2011		-	multiple
Protein-bound sulfhydryls (PBSH) (= TSH-GSH)	*2/5*		
Hoffman & Heinz, 1998		-	mallard duck, *Anas platyrhynchos*
Henny et al., 2002		↘	multiple
Hoffman et al., 2005		-	Great egret, *Ardea alba*
Kenow et al., 2008		-	Common loon, Gavia immer
Hoffman et al., 2011		↘	multiple
Thiobarbituric acid-reactive substances (TBARS) for lipid peroxidation estimation	*4/6*		

Hoffman and Heinz, 1998		**↗**	mallard duck, *Anas platyrhynchos*
Henny et al., 2002		**species dependent**	multiple
Hoffman et al., 2005		**↗**	Great egret, *Ardea alba*
Kenow et al., 2008		**-**	Common loon, Gavia immer
Hoffman et al., 2009		**-**	Snowy egret, *Egretta thula*
Hoffman et al., 2011		**↗**	multiple
Thiobarbituric acid (TBA) reaction for malondialdehyde or malonic dialdehyde (MDA)	*0/3*		
Kling and Soares, 1982		**-**	Japanese quail, *Coturnix coturnix japonica*
Ji et al., 2006		**-**	domestic ducks, shaoxing duck
Cheng et al., 2013		**-**	ducks (name not specified)
Total sulfhydryl (TSH) concentration	*2/6*		
Hoffman and Heinz, 1998		**-**	mallard duck, *Anas platyrhynchos*
Henny et al., 2002		**-**	multiple
Hoffman et al., 2005		**-**	Great egret, *Ardea alba*
Kenow et al., 2008		**-**	Common loon, Gavia immer
Hoffman et al., 2009		**↘**	Snowy egret, *Egretta thula*
Hoffman et al., 2011		**↘**	multiple

The impact of MeHg exposure on birds' physiology and behaviour has previously been reviewed (Whitney and Cristol, 2017a). Briefly, the impact of MeHg on the endocrine system (Tan et al., 2009; Wada et al., 2009), energy balance and physiological stress response can affect a bird's seasonal physiological changes and the timing of their transitions between annual cycle stages. Indeed, MeHg exposure can affect hormone concentrations such as androgens (Jayasena et al., 2011; Tartu et al., 2013), basal glucocorticoids (Adams et al., 2009; Franceschini et al., 2009; Moore et al., 2014) and stress-induced glucocorticoids (Moore et al., 2014; Wada et al., 2009), and both thyroid hormones T3 and T4 (Champoux et al., 2017; Wada et al., 2009). MeHg exposure also disrupts songbirds' metabolic balance (Gerson et al., 2019; Seewagen et al., 2022) and reduces birds' immune function (Fallacara et al., 2011; Holloway et al., 2003; Kenow et al., 2007), which could then influence other annual cycle stages. Hence, MeHg exposure could result in a mismatch between an individual's physiological state and its environment, or disturb its physiological adjustment to annual cycle stages (e.g. reproduction, moult or migration). MeHg exposure can also have long-term effects on individual's survival and reproduction (Heddle et al., 2020; Paris et al., 2018).

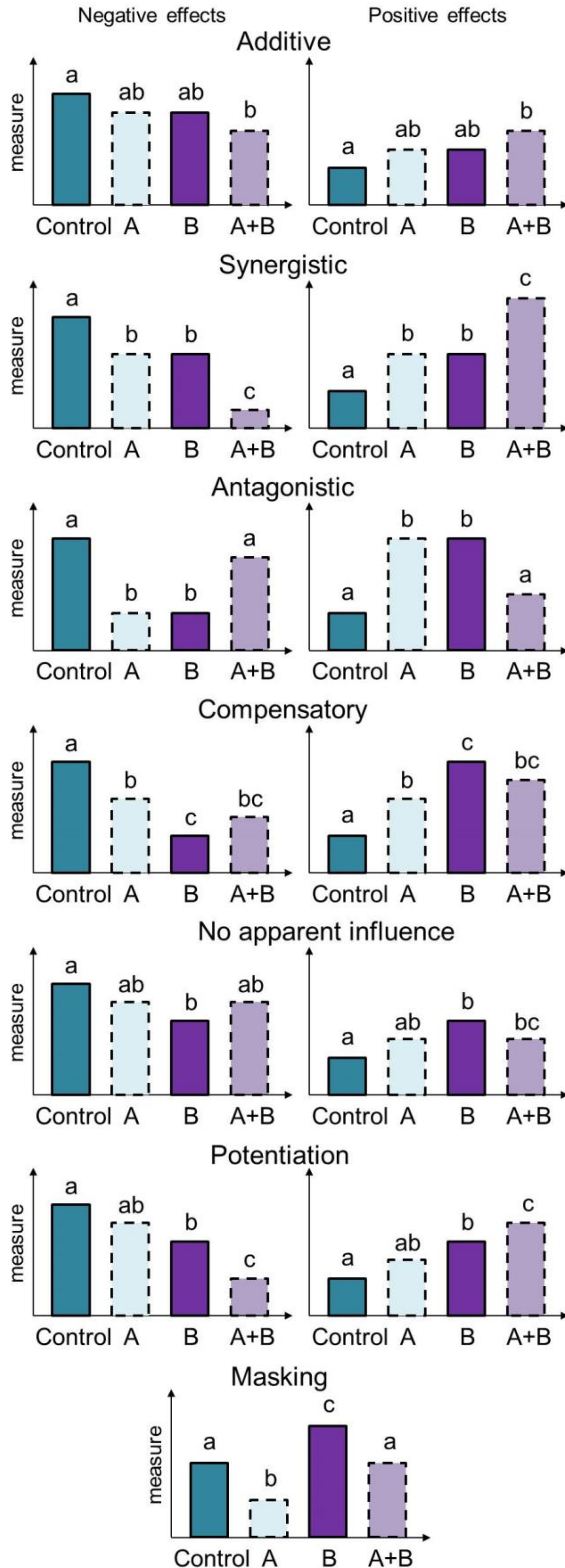
1.5. Combined exposure to MeHg and stressors and carry-over effects

1.5.1. Potential interaction between MeHg and stressors

When organisms face multiple challenges or exposures to contaminants, the outcome becomes more difficult to predict than responses to individual factors. Indeed, exposure to multiple stressors and/or contaminants could lead to antagonistic, compensatory, additive, synergistic, potentiated, or masking effects depending on the situation but could also lead to no apparent influence (all defined in figure 1.2). For example, combined exposure to pesticides and predator cues increased negative outcomes in several species (Campero et al., 2007; Coors and De Meester, 2008; Relyea, 2003). Alternatively, an effect could be not apparent either due to chemicals or stressors having different mode of action or due to the low level or potency of one contaminant resulting in no-detectable interaction

(Hernández et al., 2017; SCHER et al., 2012). There is also growing concern about a potential synergistic relationship between contaminants and stressors being a major cause of species decline (reviewed in Hooper et al., 2013; Moe et al., 2013; Wingfield and Mukai, 2009). Hence, it is important to determine how combined exposure to contaminant and stressful situation may affect organisms.

From a toxicokinetic point of view, it is still unknown if stressors may influence the bird's MeHg intake, MeHg compartmentalisation or depuration performance, but several lines of evidence suggest that this may occurs. Indeed, stressed individual may exhibit oxidative stress (Costantini et al., 2011; Hoffman et al., 2009) that may deplete antioxidant enzymes that could otherwise be used for MeHg binding and depuration. So, combined exposure to stress and MeHg may result in increased MeHg accumulation and cells damage. On the contrary, it has been suggested that food stressed bird have lower metabolic rate (Liang et al., 2015; Mckechnie and Lovegrove, 1999; Noakes et al., 2013), partially due to organ size reduction (Moe et al., 2004; Moe et al., 2005). Shorter guts could reduce the amount of MeHg gut-blood transfer. Food stressed individuals also increased or decreased their fat (Andrews et al., 2021; Cornelius et al., 2017; Fokidis et al., 2012) and muscle stores (Awerman and Romero, 2010) compared to control individuals. Although MeHg is lipophilic (log Kow = 0.41; Halbach 1985), MeHg is sequestered more easily in muscle than fat. Thus, a difference in body composition for the same exposure dose could potentially influence the compartmentalisation of MeHg within tissues. Finally, food stressed individuals grew their feathers more slowly (Andrews et al., 2021; Murphy et al., 1988; Swaddle and Witter, 1997b). This could presumably offer more opportunity for MeHg to bind with feather's keratin and hence increase the amount sequestered into the tissue. While the many years of MeHg research have not detected such a trend, to my knowledge, no study has directly tested it.



An **additive effect** occurs when combination of two or more events produce an effect equal to the sum their individual effects. This can applies to events with the same or different mode of action.
(e.g., 2+2 = 4)

A **synergistic effect** occurs when combination of two or more events produce an effect that is greater than the sum of their of individual effects.
(e.g., 2 + 2 >> 4)

An **antagonistic effect** occurs when combination of two or more events produce an effect that is lower than the sum of their individual effects. For example when the effects are canceling each other out.
(e.g., 2 + 2 << 4)

A **compensatory or inhibitory effect** occurs when an event reduces the effect of another event, producing a combined effect that is equal to one or all of the individual effects.
(e.g., 2+3 ≤ 3 and ≥ 2)

No apparent influence occurs when one event that does not have a significant effect (no different from control) does not influence the effect of a second event. In such case, the combined effect cannot be differentiated from the chemical main effect.
(e.g., 0 + 2 ≤ 2)

A potentiated effect occurs when one event that does not have a significant effect increase the effect of a second event.
(e.g., 0 + 2 > 2)

A masking effect occurs when two or more events produce opposite or functionally competing effects and diminish the effects of each other, or one overrides the effect of the other
(e.g., -2 + 2 = 0)

Figure 1.2: Visual representation and definition of the main possible outcomes from organism combined exposure to two chemicals substances or stressors (A and B). Lower case letters on top of each bar indicate the significant difference between groups. Definitions modified from Rodea-Palomares et al., (2015) to expand it to non-toxic component such as stressor exposure

From a toxicodynamic point of view, there are similarities between the physiological disruption induced by MeHg and chronic stress, suggesting that combined exposure to MeHg and stress could lead to additive or synergistic effect. This includes effects on hormones activity, oxidative stress, immunity, and behaviour. Here I reviewed 16 studies examining the results of such combined exposure in birds (Table 1-2). Most of these studies suggest that stressed individuals have a greater sensitivity to MeHg than unstressed ones. Although a few studies observed some compensatory effects (Tartu et al., 2016; Thaxton et al., 1975; Thaxton et al., 1982), or no apparent combined effect at all (de Jong et al., 2017), while some measures were not affected by one or both treatments (Table 1-2).

In general, stress can amplify the impact of contaminant exposure (Clougherty et al., 2014; Cory-Slechta et al., 2004; White et al., 2007), and MeHg exposure can alter an animal’s ability to mount an optimal stress response (Moore et al., 2014; Wada et al., 2009). For example, drought exacerbated mercury-related effects in birds (Hallinger and Cristol, 2011a; Hoffman et al., 2009) and other species (Thompson et al., 2018) and mercury exposure increased mortality risk during strong climatic events in birds (Fort et al., 2015; Roberts et al., 2014). Similarly, combined effects of MeHg and food stress on immunity are hypothesised to negatively affect seabird populations (reviewed in Sebastiano et al. 2022). Overall, combined exposure to MeHg and stressors has the potential to affect physiological performance and seasonal transitions between annual cycle stages (reviewed in Romero et al., 2017; Seewagen, 2020).

Table 1.2: Literature review of mercury and stress effects combined exposure on birds. Note that column for no apparent combined effect could contain measures that are affected by either stressor-only or MeHg-only exposure.

Authors	Species (age)	stressor	Hg exposure	Additive or synergistic effects of combined effects of stress & MeHg	Compensatory effects of combined effects of stress & MeHg	Measures with no apparent combined effect or no effect
Thaxton et al., 1975	Broiler cockerels; species not specified (chicks)	5 days of Cort intra-muscular injection	$HgCl_2$ in drinking water for 5 weeks, 2 doses at 150-300 $\mu g.mL^{-1}$	↘ body mass gain / growth at high Cort and MeHg doses	↘ body mass gain / growth at low Cort doses	-
Thaxton et al., 1982	Broiler cockerels, *Gallus domesticus* (chicks)	5 days of ACTH muscular injection	$HgCl_2$ in drinking water, 1 doses at 300 $\mu g.mL^{-1}$	↘ body weight gain / growth ↗ relative adrenal weight ↘ cholesterol in adrenal gland ↘ Bursas of Fabrisus weight	↗ Bursas of Fabrisus histopathological changes in both treatment	-plasma glucose -plasma cholesterol, -plasma protein levels -adrenal corticosteroids
Hill et al., 2008	Snowy egrets, *Egretta thula*; black-crowned night-herons, *Nycticorax nycticorax* (nestlings)	Drought years, compared to wet years (control)	Field study, bird feeding in control and contaminated sites	Complete nest failure (no young produced) in drought years for eggs at ≥ 0.8 $mg.kg^{-1}$	-	-

Hoffman et al., 2009	Snowy egrets, *Egretta thula*; Black-crowned night-herons, *Nycticorax nycticorax* (nestlings)	Drought years, compared to wet years (control)	Field study, bird feeding in control and contaminated sites	↗ number of plasma variables (10 for enzyme activity and oxidative stress, 9 other plasma constituents) that correlated with blood THg in dry years	-too numerous to describe	-too numerous to describe
Hallinger and Cristol, 2011	Tree swallow, *Tachycineta bicolor* (nestling)	Unusual high ambient temperature during early nesting period	Field study, bird feeding in control and contaminated sites	↘ fledgling %	-	-clutch size -egg volume -hatching %
Roberts et al., 2014	Eared grebes, *Podiceps nigricollis* (mostly adults, few juveniles)	Snowstorm during migration	Field study, data compared to bird pre- and post-downing	↘ fat ↘ tissue mass (liver, intestine, leg muscle) ↗ mortality (Hg concentration higher in dead downed bird than control individuals)	-	-breast muscle -sign of disease /intestinal parasite -Se liver concentration - body & tissue mass (stomach, heart)

Fort et al., 2015	Razorbills, *Alca torda;* (adults)	Winter harsh climatic event (e.g., prolonged storms collected in 2014)	Field study, data compared to dead bird from fishing bycatch, collected in 2006.	↘ body mass and body condition, ↘ tissue mass (pectoralis muscle, liver, kidney) ↗ mortality (lower liver Hg concentration but higher total Hg measured in bird dead from winter wrecks than control individuals)	-	-brain mass -Hg concentration in brain and kidney
Tartu et al., 2016	black-legged kittiwakes, *Rissa tridactyla* (adults males during incubation*)*	Cort. subcutaneous implant (25 mg pellet per 15-day release)	Field study, birds with low and high THg levels	-	-prolactin	-number of hatched eggs -number of fledglings -next year return rate
de Jong et al., 2017	Barnacle goose, *Branta leucopsis* (chicks)	social isolation	Field study, bird feeding in control and contaminated sites (coal mining area)	-	-	-body mass -haptoglobin-like activity -haemagglutination -haemolysis -basal & stress induced Cort. -nitric oxide

Scheiber et al., 2018	Barnacle goose, *Branta leucopsis* (chicks)	group isolation, individual isolation, or on-back restraint	Field study, bird feeding in control and contaminated sites (coal mining area)	↗ number of vigilance behaviour ↗ displacement with time Change in grouping behaviour with time ↗ number of escape attempts ↗ rise of fecal Cort. metabolites (unstress vs stressed period) in 2 out of 3 stressor type		-growth rate -number of stereo-typed pecks -number of distress calls -time needed to right themselves after being put on their back -baseline (unstressed) fecal Corticosterone
Still 2019	Zebra finches, scientific name not specified (adults)	Translocation	Life-long MeHg-Cys exposure dosed at 1.2 ppm w.w. in food			-basal Cort. -stress-induced (handling) Cort. -spatial memory
Zabala et al., 2020	Great egret (*Ardea alba*)	Low food availability / low water recession range / low water depth (25 years study)	Field study, birds with low and high THg levels in feather	↘ number of breeding pairs		

McLaughlin 2021	Zebra finches, *Taeniopygia guttata* (adults)	Daily 4 h unpredictable food removal during 25 days	MeHg-Cys exposure dosed at 1.2 ppm w.w. in food throughout experiment (45 days)	↘ Cort. rise with time of handling (max = 3 min)		-basal Cort. -feather Cort. -body mass change -feather mass -feather coloration -feather brightness
Zabala et al., 2021	Great egret, *Ardea alba* (nests)	Low fishes biomass / water recession (2 & 11 years study)	Field study, birds with low and high THg levels in egg or feather	↗ number of reproductive end-points affected (11 out of 12 models tested)		
Smith et al., 2022	Common eiders, *Somateria mollissima, mitiq* (adults)	Higher pre-breeding air temperatures (4 years study)	Field study, birds with low and high THg levels		earlier egg laying date ↘ incubation interruption number	
Zabala et al., 2023	Great egret, *Ardea alba (*nests)	Low food availability (11 years study)	Field study, birds with low and high THg levels in feathers	↗ early nest failures ↘ hatchling success		-clutch size -fledgling produced

Both stressors and contaminant exposure could disrupt energy acquisition and/or allocation that could lead to energetic trade-offs between traits (reviewed in Goodchild et al., 2019; Lattin and Kelly, 2020; Williams, 2018). For example, MeHg depuration and damage repairing can use energy that would otherwise be invested elsewhere (e.g., immunity, morphology, reproduction). Similarly, chronic stress could result in damage requiring repair (reviewed in Taborsky et al., 2022), additive energy use (e.g., hyperactivity; Fokidis et al., 2012; Schoenle et al., 2018) and/or reduced survival (Acevedo-Whitehouse and Duffus, 2009; Kilgas et al., 2006; Siegel, 1980). Because energy is usually a limited resource, the organism's physiological performance and energy allocation trade-off may strongly influence its survival or its reproductive success (Burton et al., 2011; Vallverdú-Coll et al., 2015; Williams, 2018). Furthermore, an unexpected energetic cost can have carry-over effects from one annual cycle stage to the other and impact animal performance (reviewed in Harrison et al., 2011). Hence, energetic expenses from stressors and/or MeHg exposure may negatively impact birds, either through direct effects during costly annual cycle stages (e.g., as moult, migration, or reproduction), or indirectly via carry-over effects across seasons.

1.5.2. MeHg and stressor carry-over effects

The definition of carry-over effects has changed with time and is still under debate. Carry-over effects have been defined to "occur when individual success in one season is influenced by events in the previous season" (Norris, 2005). These effects were later subdivided into individual-level and population-level effects that are conceptually similar, where events sustained by the individual or a change in population size in one season produces non-lethal effects that influences the individual or population *per capita* rates in the following season (Norris and Marra, 2007). This definition was further enlarged from 'events' to 'events and processes' that may involve gradual changes (e.g., feather deterioration; Harrison et al., 2011). It was then enlarged again to 'situation(s)' that can be explained by individual's previous history or experience, under the restriction that there is a clear separation period from cause to effect (e.g., treatment period, or distinct physiological states or life-stages; O'Connor et al., 2014). Overall, carry-over effects are

applicable to every annual cycle stage of a species (reviewed in Harrison et al., 2011; Moore and Martin, 2019; O'Connor et al., 2014). However, not all deviation from the population mean trait would incur a benefit or carry-over effect (Gow et al., 2019; Senner et al., 2014).

The definition of carry-over effect can be very broad and may need some refining. For example, some authors consider developmental effects on trait characteristics as carry-over effects (Moore and Martin, 2019). I suggest that carry-over effects should be separated in subcategories to differentiate between individual and population effects and between permanent and reversible effects. In this thesis, I distinguish carry-over effects from latency or developmental effects. Indeed, latency or developmental effects are caused by **irreversible** mechanisms, such as those taking place during an individual's development or aging and can be carried-over though different life-stages (Harrison et al., 2011; O'Connor et al., 2014; Pechenik, 2006). Moreover, latent adverse effects are not detected during the period of exposure to the cause of effects (Pechenik, 2006). For example, some aging-related latent effects of MeHg have been observed in humans exposed during development (Philibert et al., 2020; Weiss et al., 2002; Yorifuji et al., 2018). Hence, I define individual carry-over effects (later on 'carry-over effect') as a **reversible** process or situation inducing trait(s) change, detected in earlier stages, that has direct or indirect effects on the individual's traits or performance in a separated later period. This subcategory can then be separated between direct and indirect effects if needed, following the nuances made by Moore and Martin, (2019). Carry-over effects are often associated with negative effects of events, however, positive carry-over effects between annual cycle stages have also been suggested (Catry et al., 2013; Fayet et al., 2016).

Little is known about how the effects of MeHg and stressors might persist after exposure. Past studies indicate that elevated Cort levels (Harms et al., 2015; Imlay et al., 2019; Latta et al., 2016) or exposure to MeHg (Fort et al., 2014; Lavoie et al., 2014a; Ma et al., 2018a) can have carry-over effects. For example, there are negative carry-over effects of exposure to MeHg on breeding grounds to the survival on migration in songbirds (Ma et al., 2018a). Similarly, developmental latency effects can be induced by both MeHg

(Heddle et al., 2020; Paris et al., 2018) and stress (Gil et al., 2004; Schmidt et al., 2012) exposure. However, the physiological reversible mechanisms of MeHg exposure carry-over effects remain uncertain and, to my knowledge, no studies have examined the long-term combined effects of both stress and MeHg.

1.6. Thesis description

1.6.1. Song sparrow as model species

The model species selected in this study is the song sparrow (*Melospiza melodia*), an abundant passerine bird widespread throughout most of North America (Aldrich, 1984; Arcese et al., 2005). In particular, I used the Eastern subspecies (*M. m. melodia*) that complete short-distance migrations from Canada to southern United States (mean travelling distance of 850 km; Davis and Arcese, 1999; Kelly et al., 2019). Song sparrows mainly live in, or close to, shrub-scrub wetland habitats where they are exposed to MeHg through their diet (Jackson et al., 2011b; Keller et al., 2014; Newman et al., 2011). Song sparrows are ground foragers with an omnivore diet primarily composed of arthropods, such as grasshoppers, caterpillars, aquatic emergent insects and spiders during spring to feed the young (Girard et al., 2012; Jackson et al., 2015; Rosenberg et al., 1982). No seasonal diet shift in trophic level is observed in this species (Gagnon and Hobson, 2009). Due to its diet, habitat and abundance, this species has also been identified as a mercury sentinel (Evers et al., 2005; Jackson et al., 2015; Whitney and Cristol, 2017a).

Song sparrows also easy to catch and acclimate well to captivity, making them well studied (Arcese et al., 2005). Studies on this species particularly focus on early life stress consequence on physiology (Macdougall-Shackleton et al., 2009; Schmidt et al., 2014), immunity (Kelly et al., 2020; Kubli and MacDougall-Shackleton, 2014; Schmidt et al., 2015), moult, (Dhondt and Smith, 1980; Hudson et al., 2008), migration (Davis and Arcese, 1999; Kelly et al., 2016; Lymburner et al., 2016), neurobiology and song-control system (MacDonald et al., 2006; Mukai et al., 2009; Schmidt et al., 2013), vocal communication in mate choice (O’Loghlen and Beecher, 1999; Reid et al., 2004; Searcy, 1984), and chemical communication (Grieves et al., 2019a; Grieves et al., 2019b; Grieves

et al., 2020). These prior studies allowed me to exploit knowledge and use several well-established methods and protocols for my experiments.

1.6.2. Thesis objectives and hypothesis

My research investigated how environmental pollution could exacerbate stress and the challenge of seasonal transition for songbirds and attempted to identify potential post-exposure carry-over effects. To meet this goal, I exposed song sparrows to unpredictable food stress and/or environmentally relevant doses of MeHg, during 90 days for one summer (experiment 1) and one winter (experiment 2) and continued with post-exposure observations. This duration of 90 days was chosen to simulate a chronic exposure period that territorial birds may face during breeding or wintering seasons before other annual cycle stages like moult or migration may lead the birds out of contaminated areas. In mammals and birds, 90 day exposure duration is also considered a standard for long exposure tests (Sample et al., 1996; USEPA, 2002). This thesis is divided in 6 chapters where the following objectives are addressed:

1. Characterize the interaction between food stress and MeHg exposure from a toxicokinetic (blood and feather MeHg accumulation; Chapter 2) and toxicodynamic (synergistic, additive or compensatory physiological effects; Chapter 3 & 4) point of view.

2. Determine if stress and-or MeHg exposure affects the annual cycle timing of birds (moult, migration and reproductive onset) and identify the physiological mechanisms of action (metabolic rate, hormones levels, body condition) behind it (Chapters 3, 4 & 5).

3. Assess if carry-over effects occur and what physiological mechanisms may be most affected by them (Chapters 3, 4 & 5).

I formed three hypotheses and their respective prediction(s), corresponding to each of my objectives. First, I hypothesized that MeHg and stressors may interact in birds through their shared effects on oxidative stress, cell damage, and endocrine dysregulation.

Therefore, I predicted that birds exposed to both MeHg and food stress would show stronger physiological impairments (Figure 1.3a), either additive or synergistic, than the simple effects of stressors and MeHg separately. However, it was possible that there would be compensatory effects if these challenges have opposite effects (e.g., if MeHg increases metabolic rates while stress decreases it). Second, I hypothesized that both stress and MeHg exposure induce an energetic cost and hormonal disruption that affects birds' energy balance and seasonal transitions (Figure 1.3b). I thus predicted these challenges alone or in combination, would negatively impact the birds' metabolic rate, and body condition, and would disrupt hormone activity. I also predicted that these physiological effects would result in delay of seasonal transitions under endocrine control (moult, migratory restlessness, reproductive onset), and have negative impacts on the brain systems linked to reproduction (GnRH, song control system). Finally, I hypothesized that energetic trade-off mechanisms are active in challenging situations and under exposure, leading to long-term effects of physiological disruption (Figure 1.3b). I then predicted that these measured physiological disruptions would continue even after the end of exposure to stress or MeHg and that those disruptions could change with time due to seasonal changes.

This research strengthens our knowledge of MeHg adverse outcome pathway (AOP) and the possible durations of adverse impacts. My findings also highlight the importance of studying co-occurring stressors and contaminants when assessing potential hazards for wildlife, with the goal to ultimately improve prevention strategies and species conservation. Indeed, to my knowledge, no experimental study measured birds' energy balance to monitor MeHg influence in environmentally relevant situations (low dose exposure with stressful events) or during a post-exposure period. Additionally, while numerous measures are sensitive to MeHg exposure, no or few publications exists on MeHg effect across annual cycle stages in birds. If these are affected, it could partially explain why migratory songbird populations are decreasing.

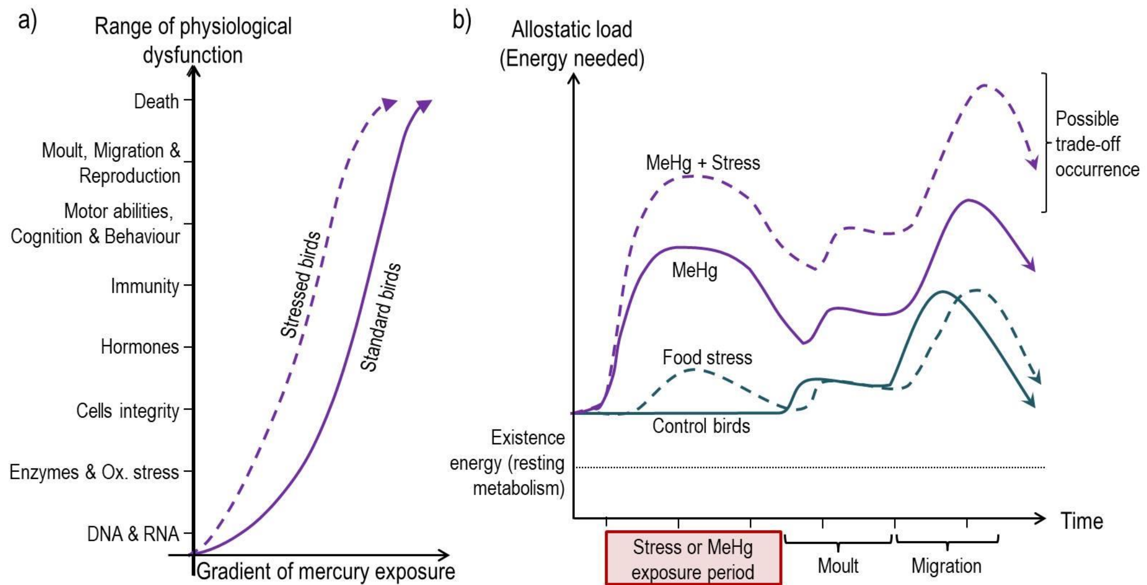


Figure 1.3: Visual representation of my PhD projects hypotheses and predictions. a) Expected sensitivity of birds when exposed to MeHg (continuous purples arrow) and both stress and MeHg (dashed purples arrow). b) Allostatic load and possible seasonal delay elicited by unpredictable food stress (dashed orange arrow), MeHg exposure and combined exposure to chronic stress and MeHg. This figure indicates the seasonal transition between summer to fall but the same hypothesis is proposed for the winter to spring transition.

1.6.3. Thesis structure

My thesis contains 6 chapters, focusing on different avian annual cycle stages where MeHg and food stress can have short- and long-term effects. In chapter 2[1], I used data collected during my first experimentation (summer exposure) to assess how MeHg load in blood changed over time and sequestered into feathers according to moult progress. I analyzed if food stress may affect MeHg loading in blood and feathers and quantified how blood concentration relates to feather sequestration. Because primary moult is

[1] Chapter 2 is published: **Bottini, C. L., MacDougall-Shackleton, S. A., Branfireun, B. A., and Hobson, K. A.** (2021). Feathers accurately reflect blood mercury at time of feather growth in a songbird. *Science of The Total Environment*, ***775***, 145739.

sequential in songbirds and feather sequestration strongly depends on blood THg levels, I predicted an increase in blood THg loading during exposure but a decrease as soon as moult started, and to observe a linear relationship between feather and blood THg, with very high levels of THg in the first-grown primary feather (P1) or in blood at time of P1 growth, and low level in the ninth primary (P9) or in blood at time of P9 growth.

In chapter 3, I assessed if MeHg and food stress exposure may disrupt song sparrow energetic performance. Data came from my first experiment where I quantified change in body condition over time, metabolic rates (basal metabolic rate, maximal metabolic rate and metabolic scope) during and after exposure, along with moult timing (onset, duration and end date) and feather quality (mass, length and mass/length ratio). My predictions for this chapter are explained in the prior section 1.6.2.

In chapter 4[2], I determined if MeHg and stress exposure may carry-over to impact migratory behaviour and the physiological mediators (Cort, T4, body condition) associated with it. These measurements were done during the first summer experiment, where I quantified hormone concentrations at different time points during exposure and post-exposure period. I assess body condition and migratory restlessness behaviour 6 weeks after the start of post-exposure period until beginning of November. My predictions for this chapter are explained in the prior section 1.6.2.

In chapter 5, I examined if winter exposure to MeHg may influence the timing of the physiological transition into reproductive phenotype in spring. Similar to my prior experiment I took several physiological and neural measures during exposure and post-exposure periods, also corresponding with a winter photoperiod and spring photoperiod respectively. I quantified body composition and hormone concentrations (Cort, T4, T3, and testosterone) at different time points throughout the experiment and collected tissues (gonad size, brain GnRH cells, neurogenesis and myelination of song control system). My predictions for this chapter are explained in the prior section 1.6.2.

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[2] Chapter 4 is published: **Bottini, C. L., Whiley, R. E., Branfireun, B. A., & MacDougall-Shackleton, S. A.** (2022). Effects of methylmercury and food stress on migratory activity in song sparrows, *Melospiza melodia*. *Hormones and Behavior*, ***146***, 105261.

Finally, in chapter 6, I integrate the findings of all the above chapters according to each of my objectives and discuss how my work advances the field of avian ecotoxicology. I also propose directions for future studies.

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Chapter 2

2. Feathers accurately reflect blood mercury at time of feather growth in a songbird[3]

2.1. Introduction

Methylmercury (MeHg) is a ubiquitous pollutant that can have deleterious effects on the physiology and survival of wildlife (Whitney and Cristol, 2017a). In birds, MeHg bound to keratin (Crewther et al., 1965) is sequestered into feathers during feather growth (i.e., moult), leading to depuration of MeHg from the blood into feathers (Bearhop et al., 2000; Carlson et al., 2014; Condon and Cristol, 2009; Fournier et al., 2002; Furness et al., 1986; Whitney and Cristol, 2017b). The growth of new feathers during moult strongly reduces mercury in blood of adult and juvenile birds (Fournier et al., 2002; Monteiro and Furness, 2001; Whitney and Cristol, 2017b), eliminating 50 to 93% of the body burden of total mercury (THg) and MeHg (Agusa et al., 2005; Braune and Gaskin, 1987; Honda et al., 1986; Lewis and Furness, 1991). Because moult follows an orderly sequence in most birds (Figure 2.1), the first primary flight feather replaced typically has a higher THg concentration than feather(s) grown later in sequence (Furness et al., 1986; Gatt et al., 2020) as the reduced mercury burden is sequentially depurated from blood and internal organs into feathers (Braune, 1987; Braune and Gaskin, 1987; Furness et al., 1986; Honda et al., 1986). This results in positive correlations between feather and blood THg concentrations (Bearhop et al., 2000; Evers et al., 1998; Kramar et al., 2019).

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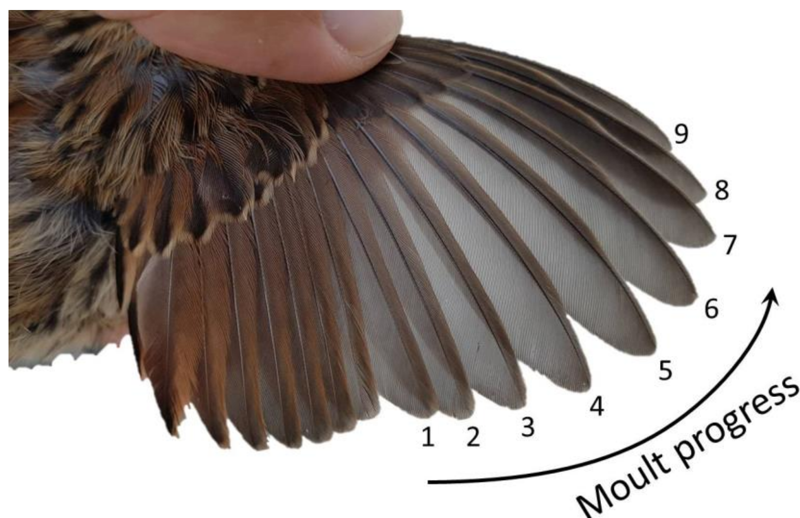

Figure 2.1: Song sparrow primary flight feathers. Arrow indicates sequential order in which the feathers moult, typically over two or three months. Photo credit: Garth Casbourn.

Measuring feather THg concentration is a frequently used tool for environmental biomonitoring due to relatively inexpensive analysis costs, non-lethal and non-invasive rapid sample collection, long-term preservation allowing historical analysis (Appelquist et al., 1985; Perkins et al., 2019; Rutkowska et al., 2018) and easily measurable high THg and MeHg concentration relative to other tissues (Honda et al., 1985; Kenow et al., 2007; Lewis and Furness, 1991). Feather THg content has been used to estimate MeHg body burden (Kim et al., 1996; Thompson et al., 1991), which can then be associated with geographic origin and/or foraging guild via stable isotope analyzes (Keller et al., 2014; Ma et al., 2021), or with physiological state via hormone or carotenoid analysis (Johns et al., 2018; Koren et al., 2012; Meillère et al., 2016).

Despite the widespread use of feather mercury for monitoring, the use of flight feathers for this purpose has been recently challenged. Indeed, variability in THg concentration among feather types from the same individual and among species, led to the recommendation to use body feathers to estimate a bird’s overall feather THg concentration (Furness et al., 1986; Peterson et al., 2019). Others observed that birds with lower THg exposures had a weaker correlation between feather and blood or other tissue THg concentrations (de Medeiros Costa et al., 2021; Edmonds et al., 2010; Evers et al.,

1998; Low et al., 2020), and an inconsistent or weak decreasing THg pattern along primary feather sequential moult (Low et al., 2020; Martínez et al., 2012; Roque et al., 2016). However, the period between the time of tissue sampling and time of feather growth could explain this finding (Edmonds et al., 2010; García-Fernández et al., 2013; Lane et al., 2020).

The objective of this study was to quantify mercury depuration rates in feathers through the progression of moult and to clarify the relationship between blood and feather mercury concentration in a wild songbird. Given that MeHg binds strongly to feather keratin during their growth, and more mercury is accumulated in feathers of individuals with higher mercury levels (Becker et al., 1994), I hypothesized that flight feather THg concentrations are mainly constrained by moulting pattern and blood THg levels at the time of feather growth. Adult song sparrows (*Melospiza melodia*) were used due to their abundance and value as a mercury sentinel species, and as a representative of migratory passerine species, many of which are experiencing rapid population declines (Rosenberg et al., 2019a). Birds were experimentally exposed to an environmentally relevant dose of MeHg and measured their blood and feather THg loading and depuration in association with prebasic moult, during which birds replace all body and flight feathers.

2.2. Materials and methods

2.2.1. Bird capture and housing

Mist nets were used to capture 54 song sparrows (11 females, 43 males) near London, Ontario, Canada (42°59'05.6"N, 81°14'43.1"W). Of these, 36 (9 females, 27 males) were captured between 8 August – 1 September 2017 and held overwinter for unrelated experiments (Grieves et al., 2019a; Grieves et al., 2019b) before being transferred to this study. An additional 18 (2 female, 16 males) were captured between 9 and 11 April 2018. Birds were housed in individual cages indoors with relative humidity of 30-70% and 20-22 °C at the Advanced Facility for Avian Research (AFAR), University of Western Ontario. Birds were kept under a simulated natural photoperiod updated every week (approximately 13 L:11 D in April – May, 15 L:9 D in June – July, 11 L:13 D in

October). Birds had access to *ad libitum* water and food (Living World Premium Mix for Budgies parakeet seed mixed with ground Mazuri small bird diet), until 16 April 2018. Then they were transitioned to a nutritionally complete synthetic agar-based diet, which became the birds' main food on 30 April 2018. This diet dry mass contained 60% carbohydrate, 13.4% protein, and 10.6% lipid (Table 2-1; made following instructions in Grieves et al., 2020). Additionally, once a week, birds were given 6 g of uncontaminated blended commercial chicken (*Gallus gallus*) eggs or two to four mealworms.

Table 2.1: Composition of synthetic agar-based diet either contaminated with 0.25 $mg.kg^{-1}$ MeHg or uncontaminated (0 $mg.kg^{-1}$MeHg)

Components	0 $mg.kg^{-1}$ (Uncontaminated)	0.25 $mg.kg^{-1}$ MeHg (Contaminated)	% dry mass	% wet mass
Corn starch (g)	300	300	30	9.7
Dextrose (g)[a]	300	300	30	9.7
Casein (g)[b]	134	134	13.4	4.3
Vitamins (g)[c]	18	18	1.8	0.6
Salt mixture (g)[d]	58	58	5.8	1.9
Celufil (g)[e]	32	32	3.2	1.0
Canola oil (g)	106	106	10.6	3.4
Agar (g)[f]	52	52	5.2	1.7
Water (mL)	1950	1950	-	62.9%
Additional water (mL)	150	149.26	-	4.8%
1000 $mg.kg^{-1}$ MeHg solution (mL)	-	0.74	-	

Product information: a) Dextrose or D-Glucose Anhydrous (Granular Powder/Certified ACS, Fisher Scientific #D16-10); b) Casein, high nitrogen, Ultrapure (Alfa Aesar by Thermo Fisher Scientific, #J12845-Q1); c) AIN-76 Vitamin mixture (MP Biomedicals, #ICN90545401); d) Briggs N Salt Mixture (MP Biomedicals, #902834); e) Cellulose fibrous (Sigma-Aldrich, #C6288); f) Agar (G-Biosciences, #RC-004).

All birds were part of a larger study involving several treatments (MeHg and unpredictable food stress exposure) balancing for sex and capture date. Briefly, for this experiment, 25 birds (6 females, 19 males) were exposed to MeHg (12 belong to MeHg-

only treatment, and 13 to combined MeHg and food stress treatment group) and blood sampled once every four weeks and their moult was monitored. An additional group of five birds (1 female, 4 males) were also exposed to MeHg, but were blood sampled every two weeks and moult was not monitored. The MeHg exposure was started on 15 or 16 May 2018. For all birds, the MeHg exposure lasted 90 days up to 13-14 August 2018, after which seven birds were euthanized. Two further birds unexpectedly died during this period. The remaining 21 mercury-exposed birds were fed an uncontaminated diet and water *ad libitum* during a post-exposure period, until they were euthanized on 31 October to 4 November 2018. For comparison, 24 non-dosed birds (12 belonged to the control group, and 12 to the food stress group) were kept in the same conditions as those above, but not exposed to MeHg. The non-dosed birds were used in the larger parallel study, but are included here to confirm that no uncontrolled contamination influenced blood THg level of exposed birds.

An agar-based diet was dosed with methylmercury chloride (5 $mg.kg^{-1}$ wet weight; Alfa Aesar, #33553) to a level of 0.19 ± 0.022 $mg.kg^{-1}$ w.w. THg (mean ± SD; concentration corrected for dry weight: 0.578 $mg.kg^{-1}$ THg). This dose is within the levels quantified in invertebrates of mercury-contaminated areas in the United States (Cristol et al., 2008; Harding et al., 2006; Newman et al., 2011). Methylmercury chloride was selected because in addition to being well studied and commercially available, its tissue accumulation is similar to the more environmentally widespread methylmercury cysteine (MeHgCys; Rutkiewicz and Basu, 2013, Varian-Ramos et al., 2017). To confirm the THg content of food 4 g of each batch of food made during the exposure period were sampled and frozen at -80 °C until analysis.

2.2.2. Moult monitoring

For the 25 MeHg-exposed birds that were blood sampled monthly, the primary flight feather moult of the right wing (see Figure 2.1) was monitored every week from July to the end of October. For each primary feather, the feather was recorded as old (not yet moulted), dropped, or as newly growing with an estimate of the growth percentage (on a

0 to 100 scale; method slightly modified from Nolan et al., 1992). Feather individual percentages were combined over the nine primaries to obtain a moult score between 0 and 900. Start date of each feather's moult was determined as the first week a missing or newly growing primary feather was observed. End date of each feather's growth was determined as the first week that the feather reached a score of 100. The entire moult period was deemed finished when the moult score reached 900. The overall moult duration was calculated as the number of weeks from primary feather moult start to finish. Some birds euthanized in August or November had not completed moult by the end of the study. For these birds, if a feather's growth was ≥70%, date of moult finish was estimated to be one week after the last measurement (n = 7 feathers) otherwise, feather mean growth date and end growth were not calculated (n = 13 feathers).

Because this study objective was to quantify the depuration and body burden for primary flight feathers, the moult and THg concentration for other feather types often collected by monitoring programs (e.g., tail or body feathers) was not precisely measured. However, the approximate progression of rectrices (tail feather) growth was recorded, allowing me to also estimate moult duration of tail feathers in nine exposed birds that did not lose tail feathers during handling. Similarly, anecdotal data were taken when head and breast feathers were at the follicle stage of growth in 13 exposed birds.

2.2.3. Feather and blood collection

For the 16 dosed birds kept for post-exposure observation starting mid-August, individuals moulted naturally pending their euthanasia at the beginning of November. Their newly grown primary feathers from the right wing were then collected post-mortem. Specifically, primary feather P1 and P2 that are the first to grow, P5 representing the middle of moult, and P7-P9 that are the last to grow (see Figure 2.1) were collected. Each feather was kept in a separate paper envelope and stored at room temperature. Feathers were weighed before being analyzed for THg content.

Blood (25-200 µL) was sampled from the brachial vein using needle puncture and heparinized microhematocrit tubes. Whole blood was frozen in microcentrifuge tubes

within 5-60 min of collection and stored at −80 °C until THg analyzes. Birds were sampled prior to MeHg exposure and then every two or every four weeks (see above).

Blood and feather mercury burden is almost entirely in the form of MeHg (Rimmer et al., 2005; Thompson and Furness, 1989a); therefore, I measured THg content and use this value as an index of bird MeHg burden. Because results of previous studies demonstrated that mercury in feathers is not influenced by external contamination from preen oil or dust (Appelquist et al., 1984; Goede and De Bruin, 1986; Jaspers et al., 2004) and because the birds were housed indoors without a contamination source for 3 months before their euthanasia, I chose not to wash the feathers before analysing them for THg.

2.2.4. Sample analysis

THg analyzes of agar food, blood and feathers were performed at the Biotron (an ISO 17025 accredited facility) at the University of Western Ontario, London, Canada using standard accredited methods for ultra-trace mercury analyzes. Samples were analyzed using a Direct Mercury Analyzer (DMA-80, Milestone Inc., Shelton, USA) following US EPA Method 7473 (EPA, 1998). For feather THg measurements, I used about 0.0057 g of feather for analysis. Due to their light weight, feathers were rolled into aluminum foil. Feathers were measured either whole for P7, P8, P9 or cut in 2 to 6 duplicated segments for P1, P2 and P5 due to their high mercury content. For food and blood THg measurements, about 0.12 g of food and 20-50 µL of blood samples were first thawed at room temperature, before vortexing them for 5 seconds prior to THg assay. All samples were placed in nickel boats for DMA analysis.

Instrument calibration was completed with certified reference material for food (DORM-4 – fish protein; National Research Council Canada), blood (certified reference CRM3 – blood standard; 50 µL of metal level 3 whole blood control; UTAK create control #44523) and feathers (IAEA-86 – human hair; MEL International Atomic Energy Agency), respectively. Laboratory quality control samples included a method blank, and aluminum foil blank when analyzing feather, an aqueous standard used for calibration check standard (CCS), and a duplicate every 10 samples. Quality assurance mean ± SD is

reported as wet weight (w.w.) concentration for blood and food or in dry weight (d.w.) concentration for feathers.

For the blood and food analysis, the mean percent recovery for the reference samples was 102 ± 4.8% (CRM3; n = 34), 99 ± 12.9% (DORM-4; n = 40), and 101 ± 2.7% (CCS; n = 16). The mean percent recovery of CRM matrix spike was 105 ± 5.5% and 103 ± 10.4% for CRM matrix spike duplicates. The mean percent recovery of food matrix spike was 92 ± 5.8% and 92 ± 6.9% for food matrix spike duplicates. The relative percent difference for duplicated spiked CRM3 was 2.9 ± 10.6% (n = 6 pairs) and 2.5 ± 1.5% (n = 6 pairs) for spiked food duplicates sample. For all samples with THg concentrations greater than 10 times the method detection limit (0.08 ng), the relative percent difference between duplicate samples was 3.1 ± 4.6 % for blood (n = 32 pairs) and 11.6 ± 6.9% for food (n = 5 pairs).

When analyzing feather samples, the mean percent recovery for the reference samples was 100 ± 4.1% (IAEA-86; n = 15), and 103 ± 4.2% (CCS; n = 5). The mean percent recovery of IAEA-86 matrix spike was 106 ± 4.6% and 105 ± 5.2% for IAEA-86 matrix spike duplicates. The relative percent difference for duplicated spiked IAEA-86 was 2.3 ± 1.7% (n = 10 pairs). For all samples with THg concentrations greater than 10 times the method detection limit (0.07 ng), the relative percent difference between duplicate samples was 11.1 ± 7.5% for feather samples (n = 10 pairs).

2.2.5. Statistical analysis

Statistical analyzes were performed using R (R Development Core Team 2017, Version 3.4.2), and a significance threshold of $\alpha = 0.05$. Data are reported as mean ± standard deviation (SD).

2.2.5.1. Blood THg

To determine blood THg accumulation and clearance rates, blood THg data was split into two parts. The accumulation phase (from day 0 to 85) was analyzed with a linear mixed effects model (*lme* with *nlme* package; Pinheiro et al., 2020) with food treatment, sex, days and the quadratic effect of days as fixed effects, and bird ID as a random effect to account for the repeated measure. For the depuration phase (from day 90 to 170) analysis, blood THg value of non-euthanized birds from day 85 was used as estimated values for day 90, assuming negligible change between those two dates. The depuration phase was analyzed with an lme model using the log-transformed blood THg value as the dependent variable. Fixed effects were treatment, sex and day and I added bird ID as a random effect. For both phases, one outlier data point was removed from the model to better fit within the requirement of normality and homogeneity. To do this, the model residual distribution was examined visually and the greatest outlier residual data point was subsequently removed. For both phases, a backwards selection method was used to sequentially drop non-significant fixed effects and the conditional R^2 of the final model was obtained via the package MuMIn.

To calculate blood half-life during the depuration phase (from day 90 to 141; August 13 to October 4), a natural log transformation was used to linearize the blood THg concentration and the slope of transformed data for each individual was calculated. This slope (k) was used to calculate the biological half-life of mercury ($t_{1/2}$), following a previously published equation (Fournier et al., 2002; Whitney and Cristol, 2017b):

$$t\frac{1}{2} = \frac{ln2}{-k} \qquad \text{Eq. (2-1)}$$

2.2.5.2. Feather THg

The change in THg concentration between adjacent feathers was analyzed to characterize changes over the moult. Data from 6 of the 89 feathers were excluded since they were dropped due to handling rather than through regular moult progression.

The time necessary for feather THg to be reduced by 50% ($t_{1/2}$) compared to the first feather grown was estimated, similar to the blood half-life calculation. A slope for each individual was calculated via a linear regression on the feather THg concentration and the number of days between the start date of first feather growth and the estimated mean growth date of each analyzed feather. Then the decrease in feather half-life since the beginning of moult was quantified using a zero-order kinetic reaction formula:

$$t\frac{1}{2} = b/2\,k \qquad \text{Eq. (2-2)}$$

Where b is the intercept of the regression and k is the slope. One bird was removed from this dataset as only two feather concentrations were available for it. All other birds had between 4 and 6 feather concentration points to calculate their slope.

Differences in THg concentration between feather positions were then assessed. A lme was used to model log-transformed feather THg concentration with sex, the primary position (P1 to P9) and food treatment as fixed effects, and bird ID as a random effect. Then, the effect of feather growth period on feather THg concentration was evaluated. In order to account for the exposure treatment effect during feather growth period, feathers were categorized as to whether 50% or more of their growth occurred during the exposure period, or whether the growth was during the post-exposure period. However, only P1, P2 and P5 had more than one feather grown in both periods, so the analysis was limited to only those feathers. This lme model contained non-transformed feather THg concentration as the dependent variable and feather growth period, the primary position (P1, P2, P5) and their interaction as fixed effects, and the bird ID as a random effect. For both models, non-significant fixed effects were dropped sequentially following the backwards selection method, and Tukey post-hoc tests was used when necessary.

2.2.5.3. Comparison of blood and feather THg

To evaluate the influence of moult progression on blood THg concentration, the blood THg matching each primary feather mean growth date was calculated first. I imputed the blood THg concentrations to match each feather mean growth date. In order to do so, I

assumed a linear change in blood THg between blood samples, and calculated the blood THg at the date of feather mean growth with the calculation:

$$\left(\frac{(THg_{After} - THg_{Before})}{Days_{After-Before}}\right) * Days_{Date-Before} + THg_{Before} \qquad \text{Eq. (2-3)}$$

Where THg_{After} is the blood THg measured after the feather mean growth date, THg_{Before} is the blood THg measured before the feather mean growth date, $Days_{After-Before}$ is the number of days between the two blood samples, $Days_{Date-Before}$ is the number of days between the estimated mean date of growth and the initial blood sample.

Most birds started to moult before the end of exposure period, thus I analyzed the relationship between blood THg and moult progress using all the birds for which I had blood and moult data, instead of only the 16 birds kept until November. I excluded data for 5 feathers for these calculations because their estimated mean growth date occurred later than the last blood sample prior to euthanasia. As well, I excluded data for 10 feathers that fell off while growing due to handling or cage contact. Finally, data from one bird was removed from analyzes because it started moult with a different feather than P1 and was euthanized early in its moult. Overall, 17 out of 151 blood data were not imputed to match feather mean date of growth.

I first wanted to assess how fast blood was depurating if moult was starting before or after the end of exposure. I run a lme model with the imputed blood THg used as dependent variable, while fixed effects were the quadratic transformation of the number of days since the beginning of moult for each bird, the feather growth period (i.e., exposure or post-exposure period) and their interaction. Bird ID was included as a random effect to account for the repeated measure. This model is different than the one published in STOTEN (Bottini et al., 2021) where I analyzed linear relationship only. The new, non-linear quadratic effect of day improves the model fit to residual homogeneity and normality assumptions, and slightly changes the interpretation of results since the original linear model did not find an interaction between the fixed effects.

Then, I run two lme models to see how imputed blood THg concentrations varied between each feather growth date. The first lme contained imputed blood THg as the

dependent variable, primary feather position and feather growth period (e.g., exposure, post-exposure) as fixed effects, and bird ID number as a random effect. In order to test for the interactive effect of feather position and growth period on THg blood concentration, a table subset was created containing only the imputed blood THg during the growth of P1 to P5, as only those had feather grown during both exposure and post-exposure. The second model had imputed blood THg as the dependent variable, the interaction between primary feather position and feather growth period as fixed effects, and bird ID number as a random effect.

The correlation strength between mercury concentration in feathers and imputed blood THg at the time of each feather's growth was examined. To do so, a Spearman correlation was run between the collected feathers' THg concentration and the blood THg levels matching each feather's mean growth date. Spearman correlation was selected over Pearson due to the non-linearity of the variable (Shapiro-Wilk test $p < 0.0001$). Regression line fit was selected by creating different models from linear regression to sixth-order polynomial fit and comparing them via their AICc and a likelihood ratio test. The models being statistically similar, a linear regression for data fit (best AICc value) was selected. Furthermore, most species in the wild tend to have lower blood THg concentration than the ones in this study (Ackerman et al., 2016a), and a prior study reported a non-linear relationship between feather and blood THg (Kramar et al., 2019). Thus, the imputed blood THg values only matching P8 and P9 dates of growth was extracted because most of the depuration had already occurred when those feathers grew (blood THg concentrations from 0.92 to 0.06 $mg.kg^{-1}$ w.w.; $n = 24$). Then, a Pearson correlation on the log-transformed blood and feather THg concentration was conducted.

2.3. Results

2.3.1. Blood mercury levels

Total mercury concentration in blood increased rapidly during the first month of experimental exposure and reached 62% of its maximum value after four weeks (Figure 2.2). Maximum blood THg levels occurred by ten weeks (Figure 2.2). After the MeHg

exposure ended (on week 12), clearance was rapid with a 71% decrease in blood THg concentration over 4 weeks, and 99% clearance by the end of the study (Figure 2.2). In comparison, 24 birds not fed methylmercury kept a low blood THg level of 0.0045 ± 0.0058 mg.kg^{-1} w.w. throughout the experiment.

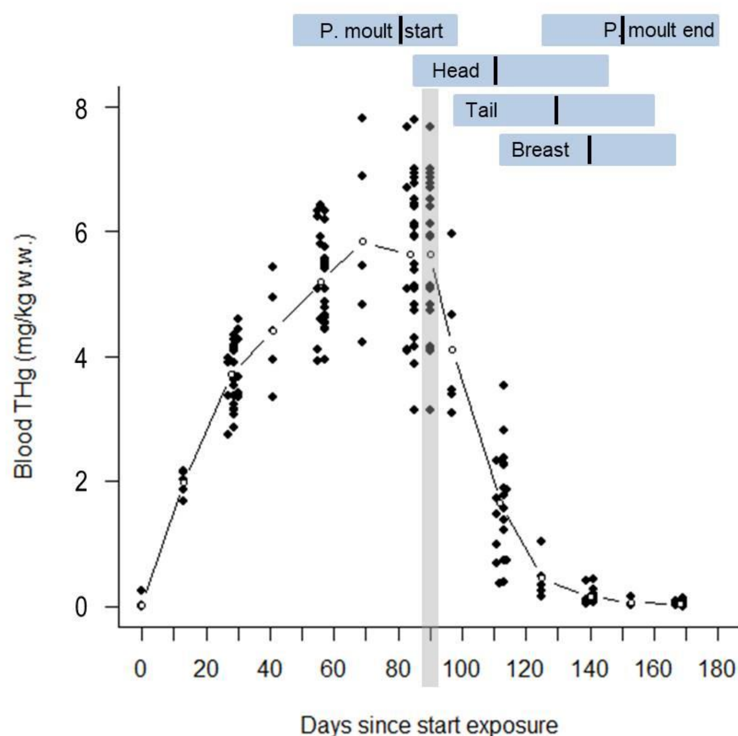

Figure 2.2: Blood THg (mg.kg^{-1} w.w.) accumulation and depuration for birds exposed during 3 months (N = 98) in summer 2018 and kept for 3 months post-exposure (N = 78). Day 0 (May 15-16) is the first day of bird exposure but with blood THg value taken prior of this date and day 173 (November 4) is day of last euthanized bird. Open circles and lines indicate the mean blood THg value for each week sampled. Filled black circles indicate individual data from each bird sampled. Vertical light grey bar indicates end of exposure on day 90 (August 13-14). Blood THg values from day 85 are double plotted to indicate they were included in both the accumulation and depuration regressions. The blue horizontal bars on top of the figure indicate the start (left side) and end (right side) primary (P.) feather moult of birds. Range of the tail moult, head and breast feathers at pin-feather stage of growth are also indicated. The vertical black line in each horizontal bar indicates the mean date of bird primary feather moult start (day 80; August 3) and end

moult (day 150; October 12) and approximate mean date of head follicle observations (day 110; 2 September), tail mean growing date (day 129; September 21) and mean date of breast follicle observation (day 140; 2 October).

Day of the study strongly explained blood THg level in both the accumulation (lme days linear: estimate = 0.15; SE = 0.006; df = 97; t = 25.97; $p < 0.0001$; lme days quadratic: estimate = −0.0010; SE = 0. 0001; df = 97; t = −14.85; $p < 0.0001$; accumulation regression: [Blood THg] = 0.07 + 0.15 × Days −0.00098 × $Days^2$; $R^2 = 0.94$; n = 30 birds) and depuration phase (lme days: estimate = −0.067; SE = 0.0017; df = 77; t = −39.12; $p < 0.0001$; depuration regression: Ln[Blood THg] = 7.75 −0.067 × Days; $R^2 = 0.95$; n = 21 birds). In exposed birds, blood mercury half-life was of 9.4 ± 1.4 days during depuration phase with moult. Neither the unpredictable food stress treatment nor sex significantly affected blood mercury levels during accumulation or depuration phases, so these effects were removed from the final lme model.

2.3.2. Feather mercury concentrations

The anecdotal data on different feather types indicate that primary feathers were the first to moult followed by head-tail feathers then by body feathers (Figure 2.3). Primary feather moult was about half-way advanced (score of 427 ± 129 mean ± SD / 900) before tail feather moult started. There was no distinct pattern between date of tail feather and head feather growth. However, breast feather growth was always observed after the tail feather started to grow (Figure 2.3).

Primary moult duration (8-11 weeks) was similar to that observed in wild song sparrows (about 3 months; Arcese et al., 2005). Feather THg decreased rapidly and reached half of peak values by 28.6 ± 4.3 days after the first feather moult was noted (Figure 2.3). This correspond to the mean growth date between P4 and P5 (P4 moult around 24.1 days and P5 around 32.9 days once moult started).

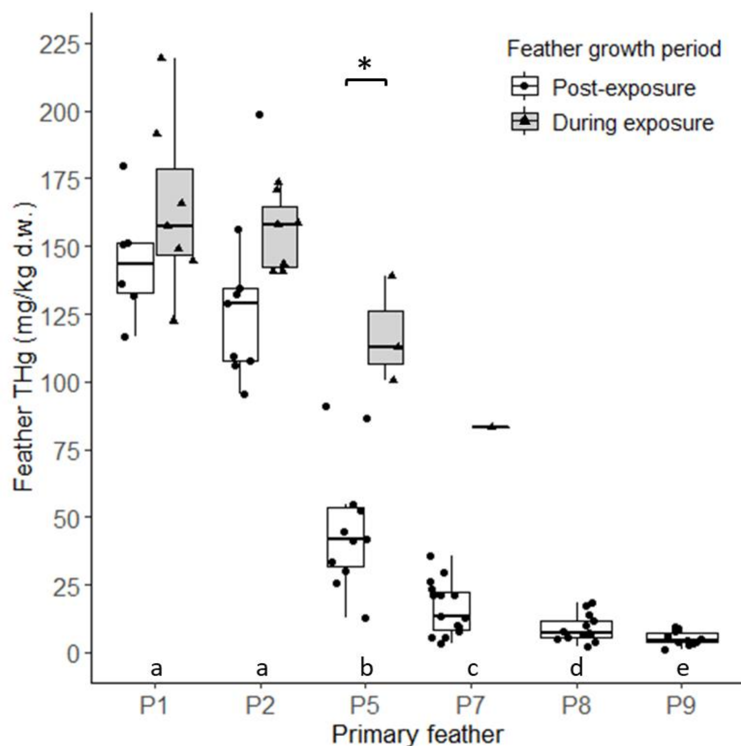

Figure 2.3: Primary feather THg concentration (mg.kg^{-1} d.w.) for feathers grown during the exposure period (grey; N = 18) or post-exposure period (white; N = 65). Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Lowercase letters on top of each feather position indicate significant differences between feathers (Tukey post-hoc test $p < 0.05$). The asterisk indicates a significant difference in feather THg concentration between growth periods for primary 5. The later analysis only includes feathers P1, P2 and P5 due to the low sample size of feathers grown during exposure.

Primary feathers differed strongly in THg concentration depending on their position (lme: $F = 284.71$; df = 5; $p < 0.0001$; Figure 2.4). Feather THg concentration showed an interaction between growth period and the primary feather position (analyzes done on P1, P2, P5 only due to limited samples size; lme on growth period × position interaction: $F = 18.98$; df = 2; $p < 0.0001$; Figure 2.4). Tukey post-hoc tests indicated that only P5 differed between growth period (estimate = −63.44; SE = 9.17; df = 22, $t = -6.92$; $p < 0.0001$) while P1 and P2 do not ($p > 0.05$). The main effect of growth period on feather THg concentration was not significant (lme: estimate = 4.48; SE = 7.40; df =22; $t = 0.61$;

p = 0.55). Other variables, part of the larger parallel study (see chapters 3 and 4), had weak to no effect on feather THg. Indeed, neither the main effect of food stress treatment nor sex influenced feather THg concentration ($p > 0.1$). However, the interaction between stress treatment and primary position was significant ($F = 3.011$; $df = 5$; $p = 0.018$), indicating that birds exposed to unpredictable food stress had slightly higher mercury sequestration into feathers throughout moulting sequence (Figure 2.4). However, taken separately, any particular feather from stressed birds contained a similar concentration than its analogue from unstressed individuals (Tukey post-hoc test $p > 0.05$).

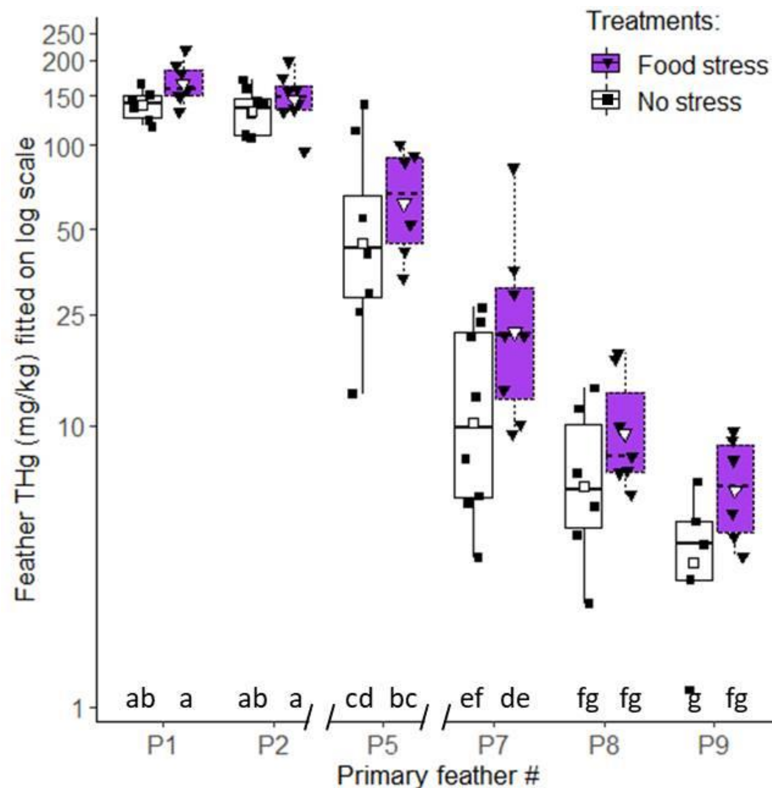

Figure 2.4: Log-scaled THg concentration (mg.kg^{-1} d.w.) of primary flight feathers of birds under unpredictable food stress (dark purple; N = 42) or no food stress treatment (light purple and dashed box; N = 41). Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Primary feather P1 is the innermost, first moulted feather while primary P9 is the outermost, last-moulted feather. Lowercase letters on top of each feather position ID indicate differences in their log-transformed THg concentration (Tukey post-hoc test $p < 0.05$; lme on primary position: treatment interaction: F-value = 3.011; df = 5; p-value= 0.018).

2.3.3. Comparison of blood and feather THg

In order to characterize the relationship between feather growth and blood THg, several tests were done. First, the temporal variation in imputed blood THg at the time the feather was grown was assessed in relation to moult progress. Blood THg concentration was influence by the interaction between days' quadratic transformation and exposure period, as well as by the main effect of exposure period and days (Table 2.2). The slope of blood THg depuration rate was stronger if feathers were grown post-exposure than during exposure (Figure 2.5).

Table 2.2: Result of lme model for imputed blood THg variation with days after moult and growth period (exposure or post-exposure). Indication of [exposure] indicates which factor group of the data is compared to the reference group (e.g., post-exposure). Final model's significant variables ($p<0.05$) are displayed in bold and italic.

Blood THg variation with days after moult and exposure period				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	2.075	0.148	13.980	**<0.001**
Days since moult start [1st degree]	***-17.078***	***0.472***	***-36.217***	***<0.001***
Days since moult start [2nd degree]	***3.179***	***0.421***	***7.553***	***<0.001***
period [exposure]	***1.010***	***0.257***	***3.929***	***<0.001***
Days since moult start [1st degree] × period [exposure]	-1.155	3.927	-0.294	0.769
Days since moult start [2nd degree] × period [exposure]	***-5.860***	***2.316***	***-2.530***	***0.013***
Random Effects				
Bird.ID τ_{00}	0.36			
Residuals σ^2	0.12			
Intraclass-correlation coef. (ICC)	0.75			
Observations (N)	134 (18)			
Marginal R^2 / Conditional R^2	0.858 / 0.965			

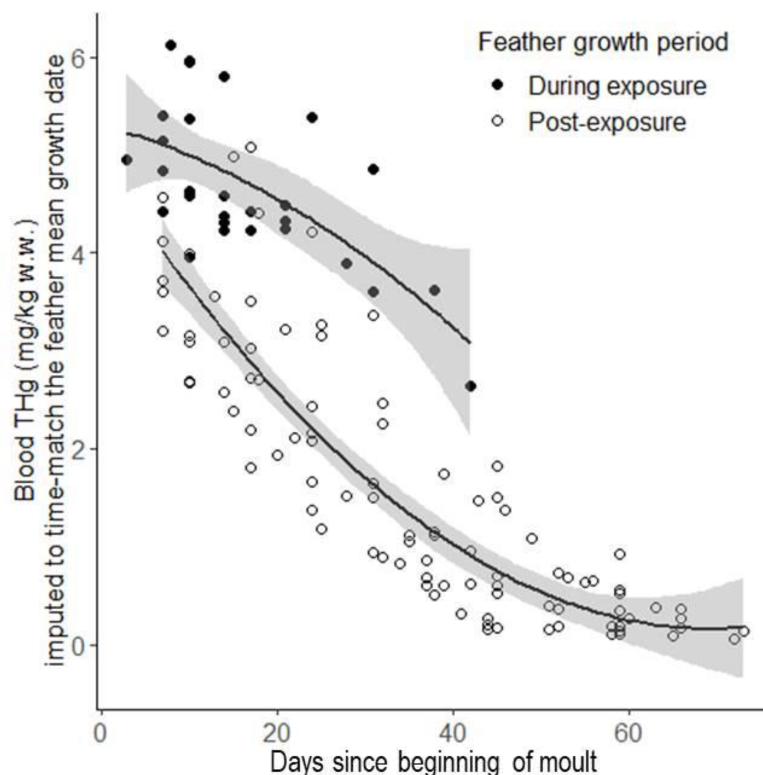

Figure 2.5: Relationship between blood THg concentration and moult progression. Imputed blood THg concentration (mg.kg^{-1} w.w.) as a function of feather mean growth date, according to the quadratic transformation of number of days since the beginning of moult. In this figure, each bird is represented several times according to its number of new feathers grown. Symbols indicate if the blood was sampled while a feather was grown during the exposure (black; N = 32; regression: $y = 5.3 - 0.024x - 0.0007x^2$; $R^2 = 0.43$) or post-exposure period (white; N = 102; regression: $y = 4.9 - 0.14x + 0.00099x^2$; $R^2 = 0.73$).

Second, the variation in imputed blood THg for each new feather grown was evaluated (Figure 2.6). In this analysis, both primary feather position (lme: F = 222.66; df = 8; $p < 0.0001$) and feather growth period (lme: estimate = 1.32; SE = 0.14; df = 107; t = 9.74; $p < 0.0001$) affected imputed blood THg concentration. The interaction effect between feather position and growth period was significant (analyzes done on feather P1 to P5 only; lme on growth period × position interaction: F = 4.35; df = 4; p = 0.0041). Post-hoc analyzes indicated that, during the post-exposure period, the growth of the first two

primaries did not alter the bird's average blood THg imputed concentration (Tukey post-hoc test, $p > 0.05$). The growth of subsequent feathers significantly altered blood THg concentration until it approached 1 mg.kg^{-1} for the growth of P7-P9 (Figure 2.6). In comparison, during exposure, it takes the growth of P4 feather to start decreasing blood THg and there was no significant decrease in blood THg between P4 and P5 (Figure 2.6).

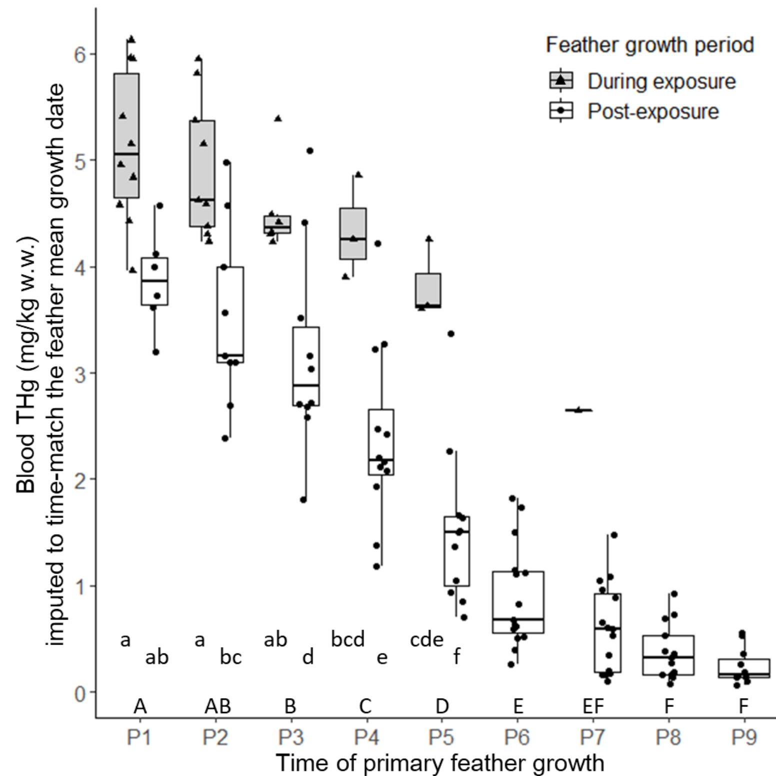

Figure 2.6: Blood THg concentration (mg.kg^{-1} w.w.; imputed to time-match feather mean growth date) changes with feather growth and accounting for exposure period during feather growth. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Colours indicate if the feather grew during exposure (grey; N = 32) or post-exposure period (white; N = 102). Primary feather P1 is the innermost, first moulted feather while primary P9 is the outermost, last-moulted feather. Upper case letters indicate the significant differences (Tukey post-hoc test $p < 0.05$) in blood THg concentration for each feather position. Lower cases letters indicate the Tukey post-hoc results for the interaction feather position: period (analysis done on feather P1 to P5 only).

Finally, the relationship between feather THg and imputed blood THg was evaluated. Across all feathers, there was a strong correlation between feather THg concentration and blood THg concentration at the mean time of feather growth (Spearman correlation: $R = 0.98$, $p < 0.0001$; regression: [blood THg] = 0.12 + 0.029 × [feather THg]; Figure 2.7). Similar results were obtained with a correlation including only samples with low THg concentrations (primary feathers P8 and P9) that are in the range that would be expected for birds living at less-contaminated sites (Pearson correlation: $R = 0.87$, $t = 8.36$, $df = 22$, $p < 0.0001$, 95% CI = [0.72, 0.94]; regression: ln[blood THg] = −1.4 + 0.99 × ln[feather THg]).

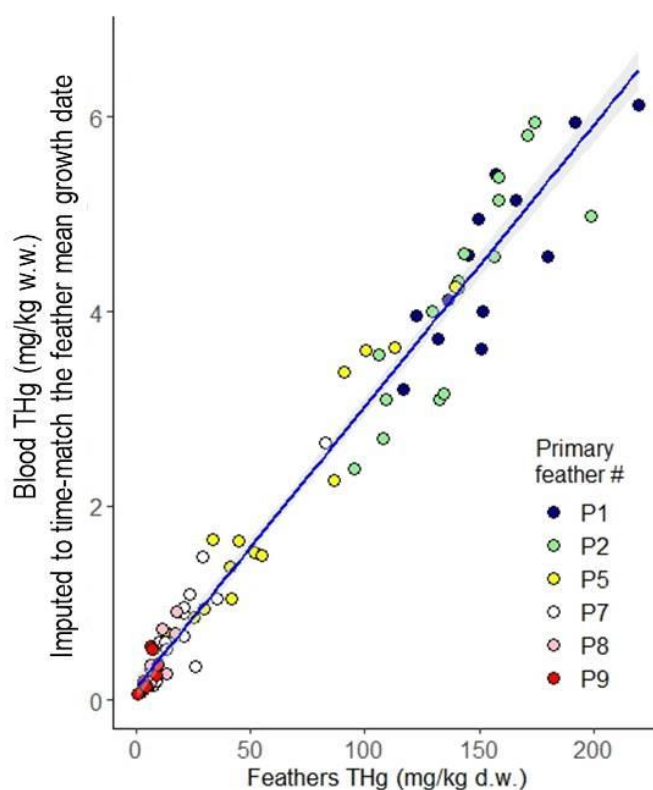

Figure 2.7: Correlation between imputed blood THg ($mg.kg^{-1}$ w.w.) and feather THg concentrations ($mg.kg^{-1}$ d.w.). Colours indicate the primary feather position (N = 83 feathers).

2.4. Discussion

In this study, I aimed to clarify if songbird feathers can be used to effectively monitor environmental mercury levels given recent criticisms that feathers show too much variability within and among individuals to be of biomonitoring utility. The results confirm that feather mercury concentration strongly reflects blood mercury content at the time the feather was grown. Previous studies have reported a decrease in blood THg during moult progression (Furness et al., 1986; Whitney and Cristol, 2017b). The remarkably strong relationship between feather THg and blood THg at the time when feathers were formed is demonstrated. This strong relationship indicated that feathers can be used as a means of monitoring environmental THg exposure to birds, but knowledge of species specify moult phenology and subsequent feather choice is critical before such an approach may be applied reliably in field studies.

The results of this study show that birds starting their moult during the exposure period had a higher blood THg level than birds starting their moult post-exposure. Thus, the former may need more time to reach similar blood THg levels as the birds starting moult in the post-exposure period. Also, the depuration slope with moult progress was stronger post-exposure, indicating that wild birds moulting in contaminated areas would also require additional time to reach low blood THg concentrations compared to birds moulting in uncontaminated areas. To my knowledge, no prior research measured blood depuration rate between different exposure groups while accounting for moult start as I did. More studies are needed to determine if other factors (e.g., variation in demethylation and elimination capacity) could influence blood depuration rate during moult independently of feather growth.

Quantifying depuration through feather sequestration compared to other depuration pathways was outside the scope of this study, but several lines of evidence indicate that depuration into feathers is a major pathway of mercury clearance from the body. First, song sparrows growing feathers during exposure had a decrease in blood THg with moult progression, and this was also observed in starlings (*Sturnus vulgaris*; Carlson et al., 2014). Second, prior work has concluded that blood THg half-life was shorter in moulting than in non-moulting birds. In this experiment, adult song sparrow blood mercury half-

life was of 9.4 days during moult. This is longer than in moulting starlings (1.1 ± 0.1 week; Whitney and Cristol, 2017b), but shorter than moulting zebra finches, *Taeniopygia guttata* (3.1 ± 0.6 week; Whitney and Cristol, 2017b), Cory's shearwater, *Calonectris diomedea* (from 38 to 65 days depending on dosing to moult time gap; Monteiro and Furness, 2001), and great skuas, *Catharacta skua* (31.5-63 days when dosing started during moult; Bearhop et al., 2000). Studies in non-moulting adult species found a longer half-life such as in Bicknell thrushes, *Catharus bicknelli*; (30 days estimated for blood; Rimmer et al., 2010), mallards, *Anas platyrhnchos* (84 days in whole body; Stickel et al., 1977), or in common loon, *Gavia immer*, chicks after plumage growth (116 day in blood; Fournier et al., 2002). These data all support the conclusion that feather moult is one of the more powerful depuration mechanisms available to birds.

Blood and feather mercury levels were tightly correlated, but this relationship is completely dependent on the time and pattern of moult. As each feather reflects a specific point in time during the depuration period and moult sequence, specific feathers can be selected to characterize mercury burden for particular periods of time (Kopec et al., 2018). Fortunately, in North American migratory songbirds, flight feather moult patterns are very well characterized and typically occur in a brief period before their autumn migration (Kiat et al., 2019b). However, a few songbird species suspend moult until arrival on the wintering grounds or have only partial moult before fall migration (Kiat et al., 2019b), increasing overall moult duration.

It is not yet fully clarified what proportion of feather mercury comes from the blood and tissue mercury accumulated during previous life stages such as breeding (i.e., an allocation of "capital"), or through diet during moult (i.e., an allocation of "income"; Cherel et al., 2018). While such a discussion is outside the scope of this paper, the present study clearly demonstrates that feathers sequester THg in proportion to the blood concentration at the time of feather growth and clarify that feather THg is strongly dependant on moult sequence (also see Gatt et al., 2020). Thus, if a bird moults outside of breeding sites (e.g., on wintering grounds or during post-breeding movement), switches diet during moult or occasionally loses a feather due adventitious replacement, the depuration pattern observed in blood and feathers will not be as linear as observed in this

experiment. Overall, refined knowledge of the location and timing of the moult sequence can better assist the interpretation of feather THg concentrations. Certainly, feather mercury data from small sample sizes should be interpreted very cautiously given the possibility of individual atypical moult, although the addition of feather stable isotope information can help eliminate some uncertainty concerning the location (and thus timing) of sampled feather growth in long-distance migratory species (Ma et al., 2021).

Finally, the choice of tissues to sample for monitoring mercury will depend on the research question, and different tissues will provide information of different time points of exposure. For example, different feather types moulted at different times will carry different temporal and spatial information according to the species moult sequence and movement patterns. Thus, feather THg among species with known different moulting sequence cannot be directly compared. As the precise timing and sequence of body feather moult is unclear for many bird species, this may limit the interpretation of mercury content of body feathers, depending on the research question. To estimate mercury exposure at non-moulting sites, blood or claw samples may better indicate contemporary or recent exposure (Low et al., 2020). Other options include sampling of nestling feathers or capture of adults twice to assess THg in feathers induced to moult by plucking.

In conclusion, flight feathers can provide an accurate and non-lethal estimate of blood mercury concentrations at the time of moult. For many North American passerines, flight feather mercury will reflect mercury exposure on their breeding grounds prior to autumn migration. Depending on the species and research question, selection of specific flight feathers can also provide information on mercury exposure earlier or later during the known moult period, so long as feathers are carefully selected and sampled. If knowledge of moult sequence and timing is limited, other tissues such as blood or claw may provide an alternative for more recent mercury exposure assessment.

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Chapter 3

3. Effects of MeHg and food stress on bird's energetic performance: metabolic rates, moult and feather quality

3.1. Introduction

Organisms regularly modify their physiology and energy balance to maintain homeostasis and adjust to predictable seasonal environmental variation. Because energy is limited, an organism's physiological performance and trade-off in energy allocation strongly influence its survival across life history stages or its lifetime reproductive success (Burton et al., 2011; Vallverdú-Coll et al., 2015; Williams, 2018). For example, food availability affects the duration of energetically costly processes such as moult in birds (Danner et al., 2015; Pap et al., 2008) and body condition was positively related to the length of the ninth primary flight feather in geese (Marmillot et al., 2016). Hence, the physiological performance of wildlife is tightly regulated to not waste energy that may be needed later. Unpredicted energetic costs can have carry-over effects from one life history stage, or annual cycle phase, to another and thus impact animal performance long after the initial cause of the cost has disappeared (reviewed in Harrison et al., 2011; Moore and Martin, 2019; O'Connor et al., 2014). For example, a reduced feather length or damaged feather resulting from costs experienced during moult could hinder flight ability and increase flight cost, and thus negatively affect a bird's energy stores during the whole year until next moult (Echeverry-Galvis and Hau, 2013; Jones and Ward, 2020; Swaddle et al., 1996). Energy management and balance is likely under strong natural selection and is associated with fitness-related traits such as movement and activity, boldness, and dominance (Arnold et al., 2021; Mathot et al., 2019).

Metabolic rates play a central role in regulating energetic performance and resource allocation. Metabolic rates vary seasonally (reviewed in McKechnie et al., 2015; Swanson, 2010) in response to energy demands and to numerous other biotic and abiotic factors (reviewed in Konarzewski and Ksiazek, 2013; White and Kearney, 2013). For example, metabolic rates change with chick rearing load (Welcker et al., 2015), and can ultimately influence survival and reproductive outcome in birds (Jimeno et al., 2020;

Rønning et al., 2016). Basal metabolic rate (BMR) corresponds to the adult minimal energy expenditure during rest in a post-absorptive state at thermoneutral temperature (McNab, 1997) and represents an animal's basic maintenance costs (Swanson et al., 2017). Maximum metabolic rate (MMR) corresponds to the maximum aerobic energy expenditure reached during locomotive exercise. Since BMR and MMR result from the activity of different tissues (e.g., internal organs and skeletal muscles respectively; (Chappell et al., 1999; Daan et al., 1990; Petit et al., 2014) they represent different ecologically relevant traits of physiological performance (reviewed in Husak and Lailvaux, 2017; Pettersen et al., 2018).

The range between MMR to BMR is an individual's metabolic scope; it represents the organism's energy pool available for aerobic activity above maintenance level, in other words the organism's capacity to increase its energy consumption for sustained physical performance (Husak and Lailvaux, 2017). Metabolic scope can be calculated either by the difference between MMR and BMR (absolute metabolic scope), or the fraction of MMR/BMR (factorial metabolic scope). Absolute scope is sometime considered more biologically relevant while factorial metabolic scope is used more often for species comparisons (Buttemer et al., 2019). Both calculations may lead to slightly different results or interpretation depending on the situation (Moe et al., 2005).

Moult is an important seasonal phase of the annual cycle in birds since moult efficiency may impact plumage quality such as feather mass (Dawson, 2004; Marzal et al., 2013; Murphy et al., 1988) and losing feathers reduces flight performance (Echeverry-Galvis and Hau, 2013; Guillemette et al., 2007; Swaddle and Witter, 1997a) or survival (Jones and Ward, 2020; Møller and Nielsen, 2018; Slagsvold and Dale, 1996). Once or twice a year, depending on species (reviewed in Kiat et al., 2019), the flight feathers are dropped and sequentially replaced, inducing an energetic cost (Fox et al., 2014; Murphy and Taruscio, 1995). Metabolic rate of birds increase during moult compared to other annual cycle stages (Lindström et al., 1993; Lustick, 1970; Portugal et al., 2007) and moult may create a 9.8-80% increase in basal metabolic rate depending on species (Buttemer et al., 2019; Murphy and King, 1992; Vézina et al., 2009). As a consequence of this cost, moulting geese may lose 22-25% of their pre-moult body mass (Fox and Kahlert, 2005;

Portugal et al., 2007) and songbirds overlapping reproduction and moult produced shorter and light feathers (Echeverry-Galvis and Hau, 2013). Energetic trade-offs and carry-over effects can thus influence birds' moult performance and feather quality.

Exposure to contaminants may disrupt energy acquisition and allocation, through depuration costs, homeostasis dysregulation, or behavioural modification (Goodchild et al., 2019). This may reduce energy available for seasonal transitions and physiological performance. For example, methylmercury (MeHg) is a neurotoxin (Henny et al., 2002; Hoffman et al., 2009; Scoville et al., 2020) that increases birds' basal metabolic rates and decreases maximal metabolic rates and metabolic scope of songbirds (Gerson et al., 2019). MeHg also affects birds' hormone levels (Champoux et al., 2017; Franceschini et al., 2009; Wada et al., 2009), moult rate (Carlson et al., 2014), and feather quality such as colour or mass (Evers et al., 2008; Giraudeau et al., 2015; White and Cristol, 2014). Exposure to MeHg is known to produce carry-over effects in birds (Heddle et al., 2020; Lavoie et al., 2014b; Ma et al., 2018a; Paris et al., 2018) but its effects on birds' energetic performance and seasonal transitions are mainly undetermined.

Similarly to MeHg, stressors may increase individuals' energetic needs and have consequences on moult and feather quality. For example, low food availability reduces feather growth rate (Bateson et al., 2021; Murphy et al., 1988; Swaddle and Witter, 1997b) or delays moult start (Dawson, 2018), while low-quality diet increases moult duration (Pap et al., 2008) or may stop moult altogether (Murphy et al., 1988; Scheiman and Dunning, 2004). In contrast, food-supplemented wild birds had an earlier moult onset or peak (Cristol et al., 2014; Danner et al., 2015; Vaucoulon et al., 1985). Also, food reduction or deprivation usually decreases basal metabolic rate (Brzęk and Konarzewski, 2001; Rønning et al., 2009; Zhang et al., 2018) but sometime increases it (Schmidt et al., 2012), and decreases maximal metabolic rate (Moe et al., 2005). Overall, chronic stress may lead to sustained glucocorticoid secretion resulting in changes in energy allocation (Fokidis et al., 2012; Schoenle et al., 2018), reducing feather regrowth rate (Romero et al., 2005) and feather quality (DesRochers et al., 2009a; Murphy et al., 1988) and lead to carry-over effects (Koren et al., 2012; Latta et al., 2016).

In this study, using a 2 × 2 experimental design, I assessed how exposure to unpredictable food stress and/or an environmentally relevant dose of MeHg affects avian physiological performance. I hypothesized that the effects of stress and MeHg exposure can accumulate and affect birds' energetic performance. I predicted that combined exposure to both stress and MeHg would have greater effect on birds' energetic performance (BMR, MMR, metabolic scope, feather quality) and seasonal transitions (body condition and moult duration) than each of these challenges alone.

3.2. Methods

3.2.1. Bird capture and housing

In this chapter, I used the same birds and experimental setup described in chapter 2. Briefly, I used 49 song sparrows (10 females, 39 males) captured in and near London, Ontario, Canada. I housed birds individually and starting on 16 April 2018, I began to feed them a handmade nutritionally complete synthetic agar-based diet (Table 2-1; Grieves et al., 2020), which became the birds' main food on 30 April 2018. Additionally, once a week, I gave the birds about 6 g of uncontaminated blended chicken eggs or 2-4 mealworms as treats.

I assigned each bird to one of four treatment groups, balancing for sex and capture date: control (n = 12: 2 females, 10 males), MeHg-only (n = 12: 3 females, 9 males), unpredictable food stress-only (n = 12: 2 females, 10 males), and combined exposure to food stress and MeHg (n = 13: 3 females, 10 males). I staggered the start of food stress and MeHg exposure by 24 h such that half of the birds in each of the four groups started treatments on day 1 and the other half started treatments on day 2. I began food stress and MeHg exposure on 15-16 May 2018 and the treatment lasted 90 days up to 13-14 August 2018. Birds undergoing unpredictable food stress treatment (food stress, or combined exposure to methylmercury and food stress) had all food removed from their cages for 3 h daily at randomly selected times during the daylight period. Birds undergoing MeHg exposure (MeHg-only and combined exposure to MeHg and stress), received the agar-based diet dosed with methylmercury chloride (5 mg.kg^{-1} wet weight; Alfa Aesar,

#33553) to a concentration of 0.19 ± 0.022 mg.kg^{-1} wet weight total mercury (THg; mean ± SD; concentration corrected for dry weight: 0.58 mg.kg^{-1} THg). Methods and results of mercury analyzis of food and blood samples are provided in chapter 2. Then 17 birds pseudo-randomly selected among treatments were euthanized for a different study. Two birds also unexpectedly died during this period. I fed the remaining 32 birds (8 in each treatment) uncontaminated agar diet and water *ad libitum* during a post-exposure period, until they were euthanized on 31 October to 4 November 2018 (Figure 3.1).

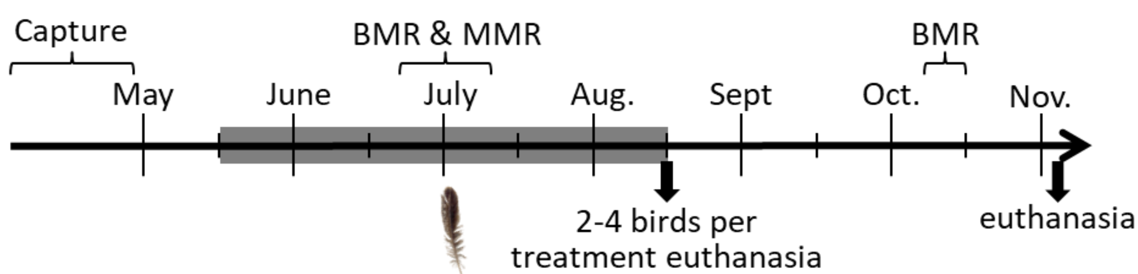

Figure 3.1: Experiment timeline of this study. Grey highlighted area indicates the period of food stress and/or MeHg exposure. The feather indicates the start of moult examination done weekly until the end of experiment. Body mass was measured every 2 weeks (not depicted). BMR = basal metabolic rate measurement; MMR = maximum metabolic rate measurement.

3.2.2. Body Condition

Before the experiment I measured each bird's tarsus length to the nearest 0.1 mm using dial calipers. I also measured each bird's body mass with an electronic balance to the nearest 0.01 g every two weeks from the beginning to the end of the experiment. I calculated body condition as body mass (g) divided by tarsus length (mm). This resulted in a continuous variable with higher values corresponding to bird with higher mass relative to its size.

3.2.3. Metabolic rates

I measured metabolic rates using open-circuit respirometry. I first measured their 'minimal' energy expenditure or basal metabolic rate (BMR) and also evaluated their maximum metabolic rate (MMR) during short term (20-30 min) exercise, following previously established methodology (Schmidt et al., 2012).

I measured the BMR of birds between 21 June to 5 July (weeks 6-8, exposure phase) and from 7 to 16 October (weeks 22-23, post-exposure phase). Each night 4-5 birds were placed in a stainless-steel chamber in a temperature-controlled cabinet at 30 °C, while a sixth chamber was used for baseline measurements. A temperature of 30 °C is within the thermoneutral zone for other species of songbirds that are similar in size to song sparrows (Root et al., 1991). In June, the birds were fasted in their home cage since 19:00, and I took body measurements beginning at 19:30 before placing the birds into a chamber. Body measurements included weight, fat and mass scores as well as body temperature (data not presented). I started to record the birds O_2 consumption and CO_2 emission around 20:15 and stopped approximately 10 h later around 6:45. Because the light schedule changed in October the birds were fasted in the chamber instead of their home cages. I took body measurement beginning at 18:30 and recording started around 19:00 to finish around 7:15 the next morning (approximately 12.25 h later). Following this, I measured each bird's body mass again and returned them to their home cage where they were left undisturbed for one full day (no food stress treatment).

To measure each bird's BMR, incurrent air was scrubbed of CO_2 and water vapour using soda lime and Drierite (W. A. Hammond Drierite Company, Xenia, OH, USA), respectively. The six sealed chambers received a constant flow of 450 $mL.min^{-1}$. Excurrent air was sub-sampled at 150 $mL.min^{-1}$. This air passed through a Drierite column to remove water before going into the CO_2 analyzer (catalogue number CA-2A, Sable Systems Las Vegas, NV, USA) and the O_2 analyzer (Sable Systems FC-1B). Gas analyzers were calibrated regularly using a standard containing 20.9% O_2 and 2% CO_2 balanced with N_2. Using a multiplexer (Sable Systems), one chamber was measured at a time for 10 min before switching to the next chamber. All instruments were connected to an analog-to-digital converter (UI-2 model, Sable Systems), which was connected to a

laptop computer. In total, each bird was measured 11 times in June and 13 times in October during their inactive period.

Data were analyzed using Expedata Warthog Systems Lab Analyst software (M. A. Chappel, University of California Riverside, Riverside, CA, USA). BMR values reported were calculated as the minimum 5 min mean of O_2 consumption throughout the measurement period. I calculated the rate of O_2 uptake (VO_2) based on equation number 10.6 in Lighton (2008) that calculates VO_2 using the data for both O_2 consumption and CO_2 production. I then converted VO_2 to watt using the equation from Lighton (2008):

$$\left((mRQ \times 5.164) + 16\right) \times mVO_2 \div 60 \qquad \text{Eq. (3-1)}$$

Where mRQ is the mean respiratory quotient (defined as the ratio of CO_2 emitted to O_2 consumed) during the 5 min. In order to compare the bird in a post-absorptive state, I made sure to discard data of metabolic rate within the first 3 h after beginning of fasting.

The same air flow system used to determine BMR was used to determine the MMR of each bird. I measured MMR between two days before up to six days after each bird's BMR measurement, between 28 June and 8 July. I measured MMR using an enclosed exercise wheel (16 × 24 cm, width × diameter) made of acrylic plastic and lined with rubber that induced birds to actively hop and hover while the wheel rotated, following prior research (Pierce et al., 2005; Price and Guglielmo, 2009; Schmidt et al., 2012). Air flowed into the wheel at a rate of 4000 $mL.min^{-1}$ and was subsampled as described above for measurements of BMR. Three small balls were placed in the wheel to prevent birds from walking. Food dishes were removed 3 h before testing (from 7:30-10:30) to ensure that birds were in a post-absorptive state. Beginning at 10:30 and finishing no later than 14:00, I measured 3-6 birds each day. Before and after each measure I weighed the bird. Once the bird was placed into the wheel I covered it and allowed the bird to acclimate for 10 min. I then spun the wheel manually to initiate exercise. The wheel was kept in constant motion so that birds were forced to hop and hover until MMR was reached (always occurred within 30 min). During the exercise I noted how active the bird was in doing the exercise. In all cases, after MMR was reached O_2 consumption decreased and then stabilized. The MMR of an individual was calculated as the maximum mean of O_2

consumption over a 1 min period expressed as watt. I then calculated the absolute metabolic scope (MMR-BMR) and factorial metabolic scope (both MMR/BMR) of each individual measured in June, to provide an estimate of aerobic capacity range.

3.2.4. Moult monitoring

I monitored birds' moult of the right wing primary flight feathers once every week from July to the end of October. For each primary feather, I recorded if the feather was an old one, had fallen, or was growing and estimated the growth percentage (on a 0 to 100 scale; method modified from Nolan et al., 1992). I combined the individual percentages over the 9 primaries to create a moult score between 0-900.

I determined the start date of each bird's moult as the first week I observed a missing or newly growing primary feather P1. I deemed the entire moult period finished when the bird moult score reached 900. For four birds, some growing feathers were lost due to handling, so moult score was estimated via the percentage growth of other growing feathers. For three birds, the last growing feathers were close to completion (≥ 70 % growth) but not fully grown at the date of euthanasia in November. In these cases, the bird's date of moult completion was estimated to be 1 week after the last measurement. Additionally, two further birds had not started to grow their last two primary feathers before euthanasia, so their date of moult completion was estimated to be 2 week after the last measurement. These estimates were based on observation that most feathers grew within 2-4 weeks. Finally, I calculated the overall moult duration as the number of weeks between moult start and finish.

3.2.5. Feather quality

I collected newly grown primary feathers post-mortem, from the right and left wing of the same 32 birds that were monitored for moult. Primary feathers P1, P2, P5, P7, P8, P9 from the right wing and P1, P5, P9 from left wing of each bird was collected after euthanasia. Each feather was kept in a separate paper envelope and stored at room

temperature. I measured the weight of each feather with an electronic scale (Sartorius CP224S) to the nearest 0.0001 g before scanning them on a flatbed document scanner (image definition: 800 dpi for right wing P2, P5, P7 and 1200 dpi for right wing P1, P8, P9 and P1, P5, P9 of left wing). I measured the curved length from the tip of the quill up to the end of rachis, following the feather rachis (Figure 3.2), up to 0.1mm, with ImageJ software and segmented line tool (scale set at either 31.4 or 47.2 pixel.mm^{-1}). Mass data from feathers for which the fuseau were still visible on the quill were discarded (N = 25/288). Feather length data were discarded if the moult data indicated that the feather had not finished growing (N = 18/288). From the remaining data, I then calculated the mass/length ratio to use in further analyzes. I also quantified the difference in length between right and left primary feathers P1, P5 and P9 as an estimate of wing asymmetry.

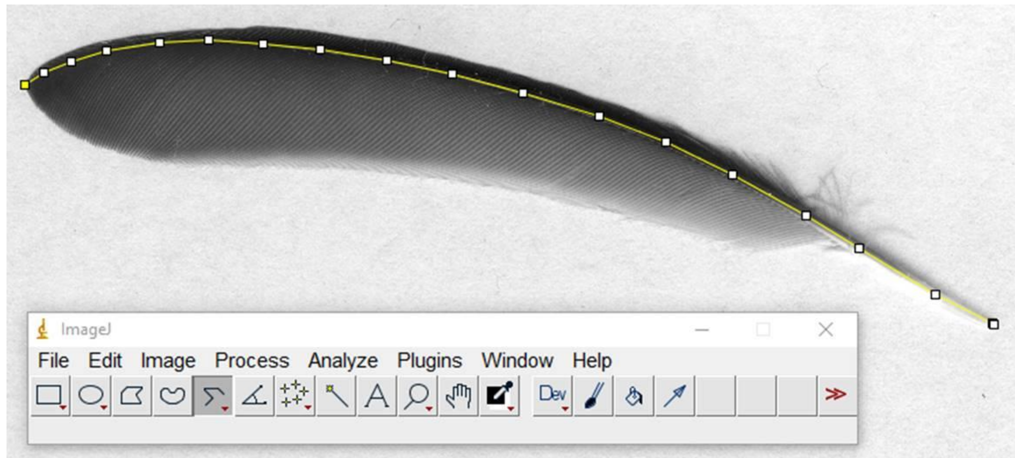

Figure 3.2: Scan (at 47.2 pixel.mm^{-1}) and measurement of a primary feather (P5) with ImageJ software. The yellow line indicates the segmented line tool use to quantify feather length.

3.2.6. Statistical analysis

All statistical analyzes were done using R program Version 4.0.3 (R Core Team, 2020), and a significant threshold of $\alpha = 0.05$. Data are reported as mean ± standard deviation (SD).

3.2.6.1. Body condition

Despite being randomly assigned, at the start of the experiment, the stress-only treatment group had slightly higher body condition than the combined stress and MeHg group. Also, females also had a lower body condition than males throughout the experiment. I hence standardized the differences by calculating the change in body condition compared to the pre-exposure time point that was then set at a zero value. Overall, the difference between raw body condition and body condition change were qualitatively equivalent.

To analyze changes in body condition over time, I used a linear mixed effect model (lme) via *lmerTest* package (Kuznetsova et al., 2017). Preliminary analysis indicated that body condition change with time was not linear and was instead better explained by a polynomial function of 5 degrees. My model hence included the triple interaction of stress, mercury and quintic transformation of time as fixed effects, as well as the variables time and bird ID as uncorrelated random intercept and slope to account for the repeated measures. This random structure was preferred over a one random intercept, correlated random intercept and slope or two random intercept random intercept structure due to preliminary checks showing a better AIC value and better model fit when compared with the *anova* function. I then removed irrelevant fixed effects by likelihood ratio test via the *drop1* function from the *stats* package (R Core Team, 2020). The model results were obtained by fitting the model with restricted maximum likelihood, and each model R^2 was extracted via the *r.squaredGLMM* function of *MuMIn* package (Barton, 2020).

3.2.6.2. Metabolic rates

In order to reduce the number of covariates in the models, I chose to study metabolic performance (for BMR and MMR) using mass-corrected data. To correct for mass I first used regression analyzes relating $\log_{10}$ transformed metabolic rate (in watts) with $\log_{10}$ transformed bird's mass at the end of measurement period. For BMR data, this linear regression was done for each time point (during exposure and post-exposure) separately. The $\log_{10}$ transformation was done since the relationship between mass and metabolic rates of animals is typically non-linear. I used this regression to calculate the scaling

equation for both BMR and MMR. Residuals of the regression were then extracted and used in further analysis.

For BMR residuals analysis, initial model residuals violated homogeneity assumptions and no data transformation or addition of fixed factor improved fit. Hence, I used a non-parametric Kruskal-Wallis test on each month separately to analyze the BMR mass corrected residual variation between treatments. If a significant effect of treatment was found, I followed-up the analysis with a Kruskal-Wallis multiple comparison test as post-hoc assessment (Dunn, 1964), since it is appropriate for groups with unequal numbers of observations, and used the default Holm methods of p-value adjustment. I also checked if month affected the BMR residuals of birds kept until November via a non-parametric Wilcoxon test. I chose this test instead of a parametric paired t-test since the homogeneity of data between month groups was not observed. Finally, several birds were still moulting at the time of the October BMR measure. I thus tested if the state of moult (finished, $n = 13$; ongoing, $n = 19$) affected the BMR residual values of those birds via a t-test since both data normality and homogeneity assumptions were met.

Before starting the analysis of MMR data, I removed four birds (two from control and two from combined exposure treatment) that were not exercising in the wheel (e.g., sliding instead of actively hopping and hovering) from the dataset. I then analyzed the MMR mass corrected residual variation with a linear model including the interaction between stress and mercury treatment, and day of measure as independent variables. Preliminary checks indicated no effects of sex or captivity history. The saturated model was then reduced to the most influencing variables via likelihood ratio test using the *drop1* function. The variable F-values were extracted via the *Anova* (type III) function while the whole model result and R^2 were extracted by the *summary* function.

Before starting the analysis of bird's metabolic scope, I used a Pearson correlation test to assess the relationship between BMR and MMR values (in watts). To be consistent with prior MMR analysis, I removed the same four birds that did not performed well during MMR measurement from analysis. I then assessed the effects of treatment on factorial metabolic scope (MMR/BMR) via linear model, with the interaction between stress and mercury, and the difference in number of days between BMR and MMR measurement as

independent effects. One data point was also removed since it skewed the model's residual normality and homogeneity. Keeping or removing this point removal led to qualitatively similar results.

I also performed a similar analysis on absolute metabolic scope (MMR-BMR). To be consistent with prior MMR analysis, I removed the same four birds that did not perform well during MMR measurement from analysis, as well as one other outlier data point. I used a linear model whose independent variables included the interaction of stress and mercury, the effect of sex, mass after MMR and mass loss during MMR (difference in mass before and after MMR measurement). Similar to factorial metabolic scope, preliminary analysis indicated an influence of day of BMR measurement that I chose to not integrate within the model. Not adding this variable does not change the interpretation of treatment effect but changed the selection of number of days as a variable in the final model. I followed the previously described protocol to do the final model's variable selection and results extraction.

3.2.6.3. Moult

Moult duration was analyzed via linear model including the interaction between stress and mercury as fixed effects as well as the sex variable as a fixed effect. Each saturated model was then reduced to the most influencing variables via likelihood ratio test using the *drop1* function. The variable F-values of each model were extracted via the *Anova* (type III) function while the whole model results and R^2 were given by the *summary* function.

Then, in a second step I checked the correlations between date of moult start, moult duration and date of moult end via spearman rank analysis. I chose a non-parametric test since these measures were not normally distributed.

3.2.6.4. Feather quality

I first assessed feather quality via their mass.length^{-1} ratio calculated for primary feathers P1, P2, P5, P7, P8, P9 of the right wing. I used lme with the *lmerTest* package to analyze feather mass.length^{-1} ratio. The original model included the triple interaction of MeHg, food stress exposure and primary feather number as fixed effects, and bird ID as a random intercept. I then removed irrelevant fixed effects by likelihood ratio test via the *drop1* function before fitting the model with restricted maximum likelihood to extract the results as described previously and the R^2 via *MuMIn* package. I also did the same analysis on feather length and mass as dependent variables.

In a second step I determined if the treatments affected the asymmetry of feather length between left and right wings. I calculated the feather P1, P5 and P9 absolute difference in length (mm) between right and left wing. This difference was then assessed within a lme model including the triple interaction of MeHg, food stress exposure and primary feather number as fixed effects, and bird ID as a random intercept. I then removed irrelevant variables as described above.

3.3. Results

3.3.1. Body condition

I first assessed how body condition changed with time and experimental treatments. MeHg exposure was not retained as a fixed effect since it did not significantly influence changes in body condition ($p = 0.49$) or in interaction with time ($p = 0.081$ for all birds, $p = 0.21$ for males only). The final lme model included a non-significant main effect of stress, the main effect of quintic time and the interaction between time and stress treatment (Table 3-1). Birds showed a similar change in body condition with time, except on weeks 16-20, during the post-exposure period, when the increase in body condition during moult had a higher peak in stressed birds than in unstressed birds (Figure 3.3).

Table 3.1: Results of the final lme model of body condition change since pre-experimental values. Square brackets (e.g. stress [Y]) indicate which level within a factor is compared to the reference group (e.g., unstressed birds). Final model's significant variables ($p<0.05$) are displayed in bold and italic.

Body condition change model				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	***0.026***	***0.007***	***3.93***	***<0.001***
stress [Y]	0.0092	0.009	0.99	0.322
Time [1st degree]	***0.54***	***0.086***	***6.23***	***<0.001***
Time [2nd degree]	***0.13***	***0.038***	***3.54***	***<0.001***
Time [3rd degree]	-0.020	0.036	-0.54	0.589
Time [4th degree]	0.028	0.036	0.77	0.440
Time [5th degree]	***0.16***	***0.036***	***4.55***	***<0.001***
stress [Y] × Time [1st degree]	0.013	0.120	0.11	0.915
stress [Y] × Time [2nd degree]	***-0.16***	***0.053***	***-3.038***	***0.002***
stress [Y] × Time [3rd degree]	***-0.10***	***0.051***	***-2.024***	***0.043***
stress [Y] × Time [4th degree]	-0.074	0.051	-1.46	0.145
stress [Y] × Time [5th degree]	-0.0036	0.051	-0.071	0.944
Random Effects	*Variance*	*Std. Dev.*		
Bird.ID τ_{00}	0.00051	0.022		
Bird.ID per Time τ_{11}	<0.0001	0.0020		
Residuals σ^2	0.00063	0.025		
ICC	0.44			
$N_{\text{birds ID}}$ (Observations)	49 (525)			
Marginal R^2 / Conditional R^2	0.27 / 0.75			

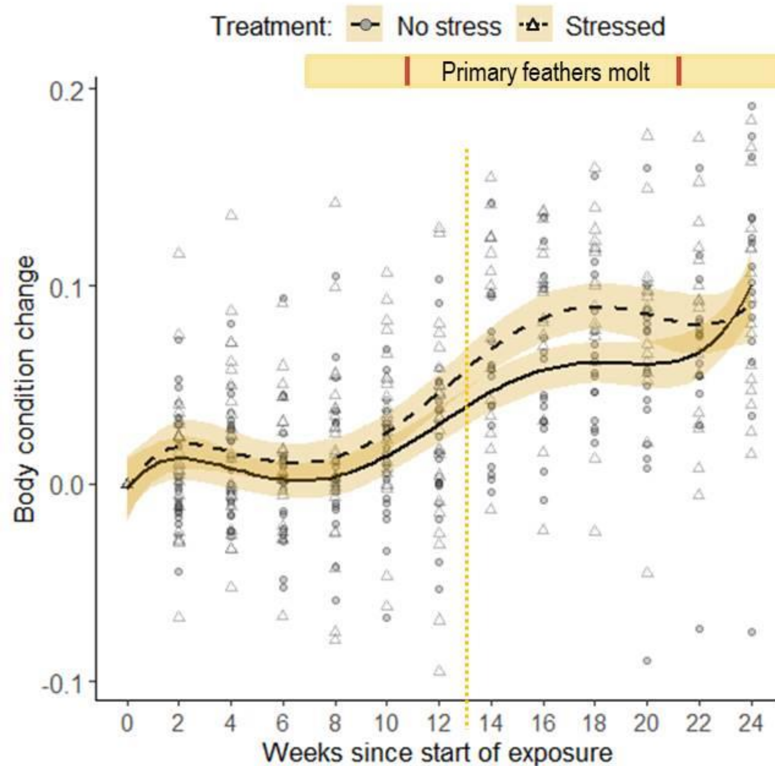

Figure 3.3: Change in body condition (mean ± se of mass.tarsus^{-1}; g.mm^{-1}) according to time (weeks) and food stress treatment (pooled across MeHg treatments). Time point 0 correspond to pre-experiment body condition measured on 14-15 May. The yellow dotted vertical line indicates the end of treatment exposure and euthanasia of 2-4 birds per treatment. The horizontal yellow bar indicates the start and end of the moult period with vertical red lines indicating the mean date of moult start (3 August) and moult end (12 October). Lines were fitted via linear model with a polynomial function of 5 degrees. Solid line and filled circles (n = 16 by end of experiment) indicate birds with no food stress dashed line and open triangles (n = 16) indicate birds with food stress.

3.3.2. Metabolic rates

The basal metabolic rate to mass scaling equation was $Y = 0.29M^{0.64}$ during exposure (log_{10} mass 95% CI = 0.36 - 0.93) and $Y = 0.37M^{0.47}$ in October (log_{10} mass 95% CI = -0.033 - 0.96). Treatment affected BMR residual during the time of exposure (Kruskal-

Wallis test: chi-squared = 10.99, df = 3, p = 0.012; Figure 3.4a) but not after 2 months post-exposure in October (chi-squared = 2.065, df = 3, p = 0.57). During exposure the BMR residuals from birds in the stress-only treatment (mean ± SD: -0.023 ± 0.019 watt, n=11) were lower than the control treatment (0.0086 ± 0.026 watt, n=11, Kruskal-Wallis multiple comparison: p = 0.034) and MeHg-only treatment (0.012 ± 0.023 $watt.s^{-1}$, n=10, p = 0.016), but not significantly lower than the combined MeHg and stress treatment (0.0038 ± 0.024 watt, n=12, p = 0.078). No difference was observed among other treatments groups (p > 0.1; Figure 3.4a). There was no effect of month on the BMR residuals of birds kept until the end of experiment (Wilcoxon signed rank test: V = 262, p = 0.98). Furthermore, moulting state did not influence BMR residuals of birds in October (t-test: t = -1.59, df = 27.4, p = 0.12). As a follow-up analysis, I run an lme only on the birds kept until end of November, including the interaction of mercury × stress × month and mass loss during BMR measurement. This model met statistical assumptions and gave similar results to the 2 Kruskal-Wallis and Wilcoxon test separately and resulted in significant main effects of each variable but no interaction.

The MMR scaling equation was $Y=0.39M^{1.08}$ (log_{10} mass 95% CI = 0.58 - 1.58). The birds' MMR residuals during exposure were not affected by treatment (variables excluded from model with all p > 0.1; Figure 3.4b), however it was negatively influenced by the day of measure (lm: $F_{1,37} = 8.47$, p = 0.011). The last day of MMR measurement appeared to drive this relationship since if these data points are removed, day was no longer significant (p = 0.33). Furthermore, the model with days did not explain the data spread well (lm model: $F_{1,37} = 7.16$, residuals SE = 0.034; adjusted $R^2 = 0.14$, p = 0.011).

To better understand how MMR values relate to BMR values, I assessed the correlation between theses variables. Birds' BMR (in watt, not residual) was not related to birds' MMR values (Pearson correlation: t = 1.94, df = 36, r = 0.31, 95% CI = -0.014-0.57, p = 0.060). However, if the four birds excluded for not exercising were kept in the dataset, a positive relation was observed (Pearson correlation: t = 2.15, df = 40, r = 0.32, 95% CI = 0.020-0.57, p = 0.038), suggesting that BMR and MMR covary.

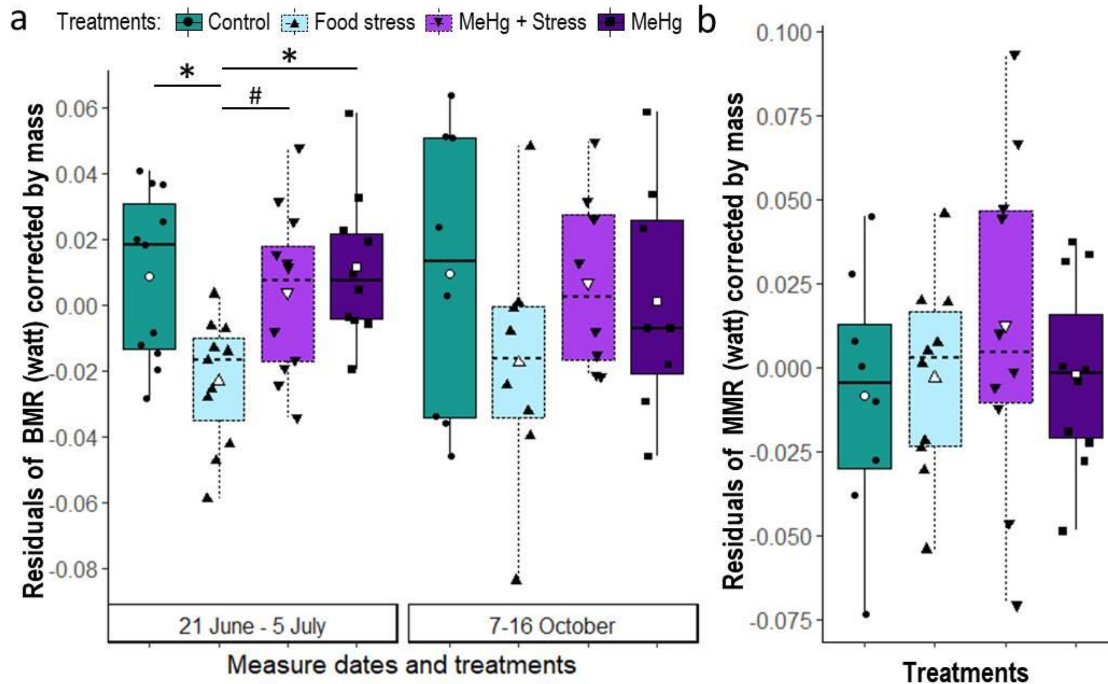

Figure 3.4: Basal and maximal metabolic rate (watts) results. a) Treatments effect on residuals of basal metabolic rate corrected by mass compared between exposure and post-exposure periods. b) Treatments effects on maximal metabolic rate during exposure period. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: control (dark blue with circles), food stress (light blue with triangles), combined MeHg and food stress (light purple with inverted triangles), MeHg (dark purple with squares). The white symbols indicate the mean metabolic rates for each treatment group. Asterisk symbols indicate difference between treatment: # $p < 0.01$ and * $p < 0.05$.

The bird's factorial metabolic scope (MMR/BMR) final model included the effect of stress (lm: $F_{1,34} = 1.74$, $p = 0.047$; Figure 3.5a) and number of days between measurement of BMR and MMR (lm: $F_{1,34} = 2.53$, $p = 0.018$; Figure 3.5b). Factorial metabolic scope increased in food stressed birds (7.47 ± 0.71 watt, n = 19) compared to unstressed birds (7.05 ± 0.66 watt, n = 18) and in birds with the largest number of days between BMR and MMR measures. Although statistically significant, this model explained a lower proportion of the data (lm model: $F_{2,34} = 5.038$, residuals SE = 0.64; adjusted $R^2 = 0.18$, $p = 0.012$) than the absolute metabolic scope model (see below).

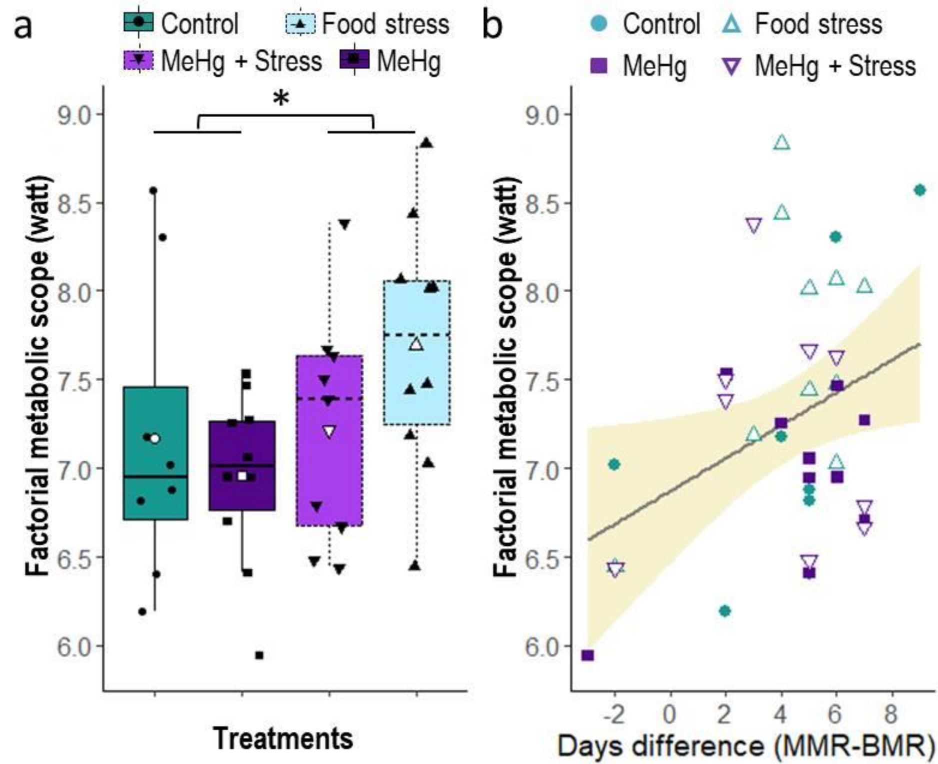

Figure 3.5: Factorial metabolic scope (MMR/BMR in watts) results. a) Effect of treatments on factorial metabolic scope. b) Effect of number of days between MMR and BMR measures on factorial metabolic scope. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: control (dark blue with circles), food stress (light blue with triangles), combined MeHg and food stress (light purple with inverted triangles), MeHg (dark purple with squares). The white symbols indicate the mean metabolic rates for each treatment group. Asterisk indicates a significant difference between treatments ($p < 0.05$).

The bird's absolute metabolic scope (MMR-BMR) final model did not include the effect of stress, MeHg exposure or sex (all $p > 0.1$; Figure 3.6). Absolute metabolic scope was influenced by birds' mass (lm: $F_{1,34} = 14.36$, $p < 0.001$), and mass loss during MMR measurement (lm: $F_{1,34} = 4.36$, $p = 0.044$). The model explained a substantial proportion of the data (lm model: $F_{2,34} = 9.36$, residuals SD = 0.24; adjusted $R^2 = 0.32$, $p < 0.001$). I

also verified that birds' mass after MMR and mass loss during MMR were not correlated (Spearman correlation: S = 6264.3, rho = 0.26, p = 0.12).

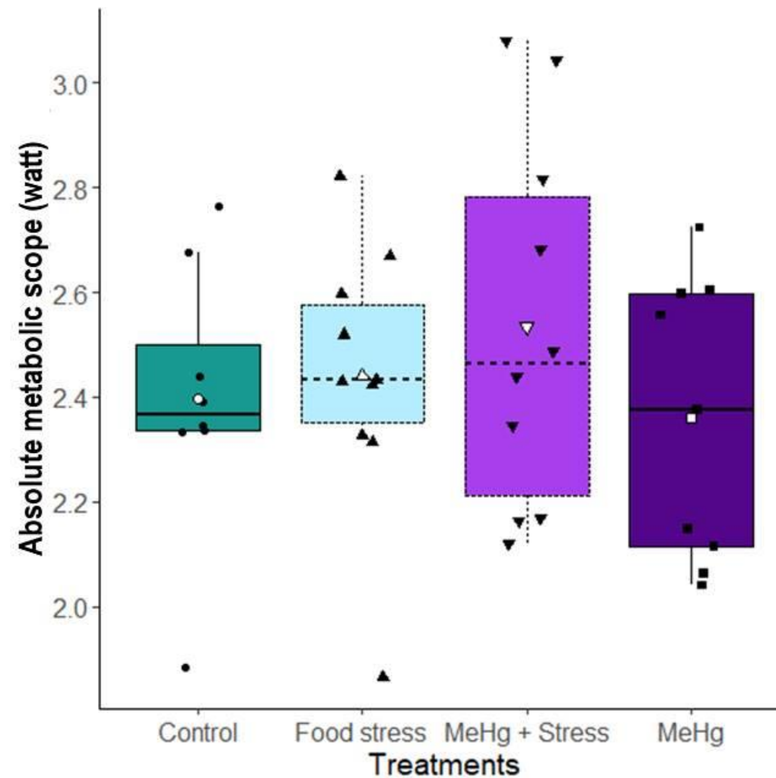

Figure 3.6: Variation of absolute metabolic scope (MMR-BMR in watts) according to treatment groups. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: control (dark blue with circles), food stress (light blue with triangles), combined MeHg and food stress (light purple with inverted triangles), MeHg (dark purple with squares). The white symbols indicate the mean absolute metabolic scope for each treatment group.

3.3.3. Moult

The final model for moult duration included the effects of MeHg exposure (lm: $F_{1,29}$ = 9.75, p = 0.004; Figure 3.7) and sex (lm: $F_{1,29}$ = 5.75, p = 0.023). MeHg exposed birds took longer (10.50 ± 1.79 weeks; n = 16) to moult compared to unexposed birds (8.88 ±

1.09 weeks; n= 16), while females (11.20 ± 2.39 weeks; n = 5) took longer to moult than males (9.41 ± 1.39 weeks; n= 27). The model was significant (lm model: $F_{2,29} = 8.46$, residuals SE = 1.38; adjusted $R^2 = 0.33$, $p = 0.0013$). Despite the significant effect on moult duration, similar analysis on moult start and end demonstrated no treatment effect on these measures (all $p > 0.05$).

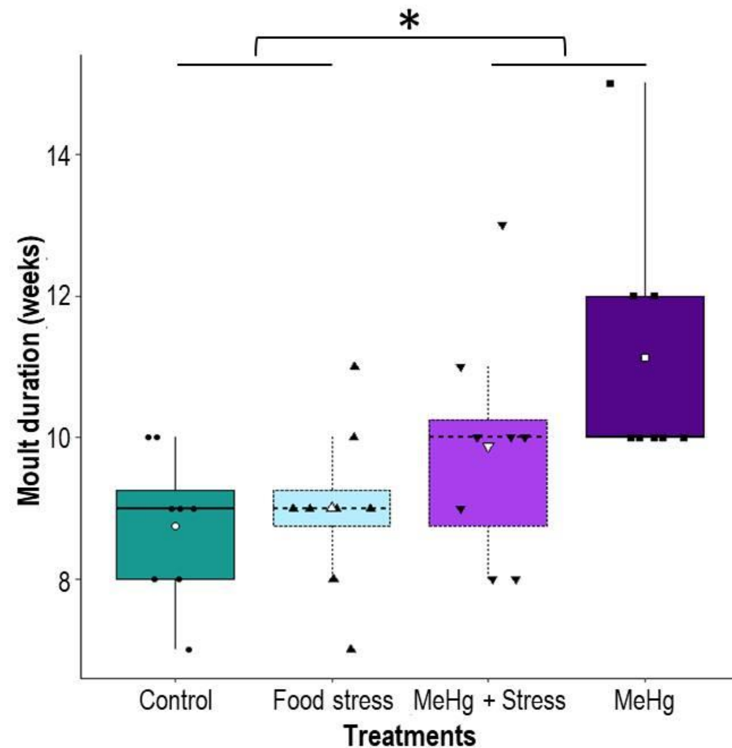

Figure 3.7: Effect of food stress and MeHg exposure treatments on moult duration (in weeks). Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: control (dark blue with circles), food stress (light blue with triangles), combined MeHg and food stress (light purple with inverted triangles), MeHg (dark purple with squares). The white symbols indicate the mean moult duration for each treatment group. The asterisk symbol indicates linear model difference between MeHg treatment groups ($p < 0.05$).

I also assessed how moult start, duration and end date are related to each other. Spearman correlations indicated that moult start date is positively correlated with moult end date

(Spearman: S = 1773.1; rho = 0.68; p <0.001; Figure 3.8a), but not with moult duration (Spearman: S = 6914.9; rho = -0.27; p = 0.14; Figure 3.8b), while moult duration was positively correlated with moult end date (Spearman: S = 3085.3; rho = 0.44; p = 0.013; Figure 3.8c).

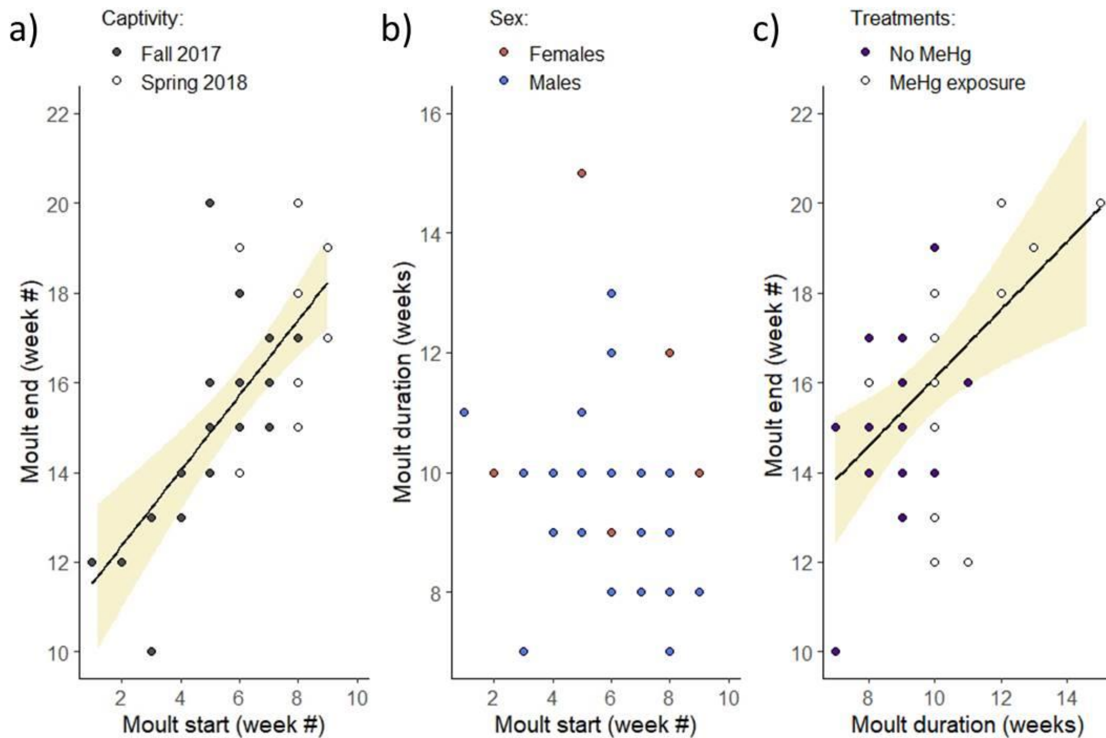

Figure 3.8: Spearman correlations between moult measurements. The regression line and its standard error indicate a significant linear relation between measures. a) Correlation between moult start date and moult end date. To illustrate the influential effect of bird's captive history, grey symbols indicates birds caught in fall 2017 and white symbols for birds captured in spring 2018. b) Correlation between moult start date and moult duration (in weeks). To illustrate influential effect of bird's sex, blues symbols indicate male and red symbols female birds. c) Correlation between duration (in weeks) and moult end date. Symbols illustrated influential effect of MeHg exposure with white circles indicating unexposed groups (control and stress-only treatment) and purple symbols indicating exposed individuals (from MeHg-only or coexposure treatment).

3.3.4. Feather quality and wing asymmetry

I also explored whether exposure to MeHg and food stress affected feather quality. The final selected model of feather mass.length^{-1} ratio included the non-significant main effect of MeHg, but a significant difference between primary feathers, and a significant interaction between MeHg and primary feather (Table 3.2). The significant interaction indicates that the way feather mass.length^{-1} ratio varied across primary feathers differed between MeHg exposed and non-exposed birds, with specific inner primaries having greater mass.length^{-1} ratio in MeHg exposed birds (Figure 3.9). Neither stress nor the interaction between stress and primary feather were retained in the final models ($p > 0.1$). Very similar results were also observed for feather mass and length (Table 3.2).

The asymmetry in length of feathers P1, P5, and P9 between the right and left wing were not influenced by any treatment or feather type (lme: all $p > 0.1$). The final selected model was a null linear model.

Table 3.2: Comparison of final lme model results of song sparrow's feather mass.length-1 ratio, mass and length, selected after variable removal via drop1 function. Indication of [Y] or [#] signal which factor group of the data (e.g., group exposed to mercury, primary feather #) is compared to the reference group (e.g., unexposed birds, primary P1). Marginal R^2 indicates the variance explained only by fixed effects and conditional R^2 indicates the variance explained by the fixed and random effects. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

	Feather mass.length^{-1} ratio**model**		**Feather mass model**		**Feather length model**	
Predictors	*Estimates (95% C.I.)*	*Statistic (p-value)*	*Estimates (95% C.I.)*	*Statistic (p-value)*	*Estimates (95% C.I.)*	*Statistic (p-value)*
(Intercept)	***0.12 (0.12 – 0.13)***	***72.151 (<0.001)***	***6.270 (5.80 – 6.745)***	***25.87 (<0.001)***	***52.67 (51.28 – 54.09)***	***73.52 (<0.001)***
sex [M]			***0.778 (0.32 – 1.24)***	***3.34 (0.002)***	***3.57 (2.22 – 4.92)***	***5.20 (<0.001)***
mercury [Y]	0.00040 (-0.004 – 0.005)	0.17 (0.866)	0.141 (-0.22 – 0.51)	0.76 (0.45)	0.71 (-0.38 – 1.79)	1.27 (0.21)
feather [2]	-0.0020 (-0.004 – 0.001)	-1.35 (0.181)	0.062 (-0.10 – 0.23)	0.73 (0.46)	***1.25 (0.72 – 1.77)***	***4.67 (<0.001)***
feather [5]	***0.013 (0.011 – 0.016)***	***10.91 (<0.001)***	***1.420 (1.25 – 1.59)***	***16.35 (<0.001)***	***4.80 (4.27 – 5.32)***	***17.96 (<0.001)***
feather [7]	***0.018 (0.016 – 0.021)***	***15.12 (<0.001)***	***1.406 (1.24 – 1.57)***	***16.51 (<0.001)***	***2.69 (2.17 – 3.21)***	***10.075 (<0.001)***
feather [8]	***0.022 (0.019 – 0.024)***	***17.69 (<0.001)***	***1.20 (1.024 – 1.37)***	***13.74 (<0.001)***	*-0.18 (-0.72 – 0.35)*	*-0.66 (0.51)*
feather [9]	***0.027 (0.024 – 0.030)***	***20.32 (<0.001)***	***0.46 (0.28 – 0.64)***	***4.92 (<0.001)***	***-6.85 (-7.40 – -6.31)***	***-24.62 (<0.001)***
mercury [Y] × feather [2]	***0.0041 (0.001 – 0.007)***	***2.37 (0.019)***	***0.31 (0.069 – 0.52)***	***2.53 (0.013)***	0.64 (-0.099 – 1.38)	1.70 (0.092)

mercury [Y] × feather [5]	0.0016 (-0.002 – 0.005)	0.90 (0.37)	0.12 (-0.13 – 0.363)	0.94 (0.35)	0.19 (-0.57 – 0.94)	0.48 (0.63)
mercury [Y] × feather [7]	0.0015 (-0.002 – 0.005)	0.87 (0.39)	0.070 (-0.17 – 0.31)	0.57 (0.57)	-0.13 (-0.87 – 0.62)	-0.33 (0.74)
mercury [Y] × feather [8]	-0.00043 (-0.004 – 0.003)	-0.25 (0.81)	0.014 (-0.23 – 0.26)	0.12 (0.91)	0.25 (-0.51 – 1.017)	0.65 (0.52)
mercury [Y] × feather [9]	-0.0017 (-0.006 – 0.002)	-0.88 (0.38)	-0.20 (-0.47 – 0.072)	-1.43 (0.15)	-0.66 (-1.45 – 0.13)	-1.63 (0.11)
Random Effects	*Variance*	*Std.Dev.*	*Variance*	*Std.Dev.*	*Variance*	*Std.Dev.*
Bird.ID τ_{00}	0.000036	0.0060	0.22	0.46	1.86	1.37
Residuals σ^2	0.000012	0.0034	0.05	0.24	0.57	0.76
Intraclass-correlation coef. (ICC)	0.75		0.79		0.77	
N $_{birds\ ID}$ (Observations)	32 (175)		32 (175)		32 (181)	
Marginal R^2 / Conditional R^2	0.681 / 0.922		0.623 / 0.920		0.850 / 0.965	

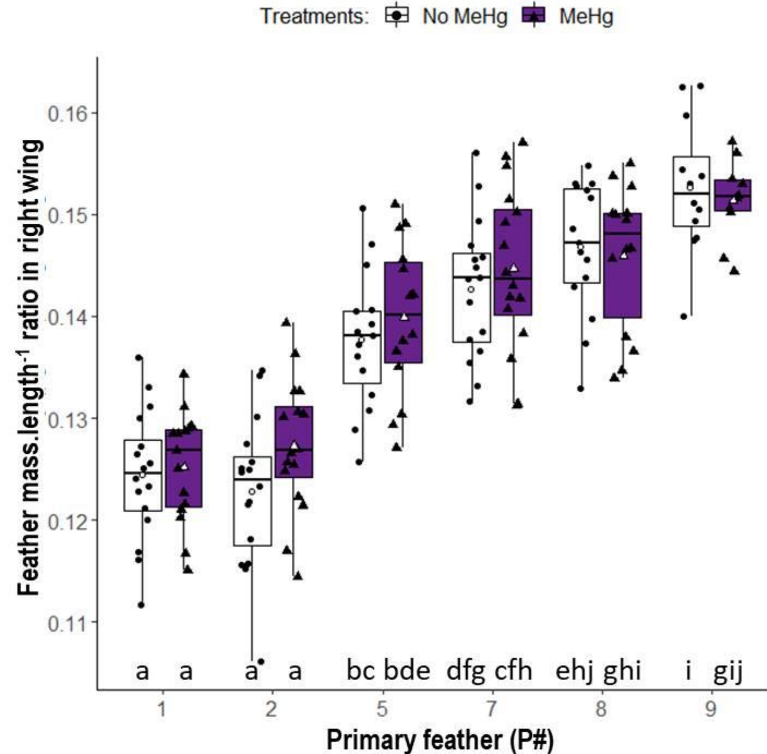

Figure 3.9: Effect of MeHg exposure on right wing's primary feathers mass.length^{-1} ratio ($\mu g.mm^{-1}$). P1 is the innermost primary and usually the first primary feather being moulted, while P9 is the outer feather. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: unexposed birds (regrouping control and stress-only treatments, indicated by circles on white box) and MeHg exposed birds (regrouping MeHg-only or coexposure treatments, indicated by triangle on purple box). The white symbols indicate the mean body condition for each treatment group. The figure's lower case letters indicate significant difference between groups (Tukey post-hoc test $p < 0.05$).

3.4. Discussion

In this chapter, I aimed to assess if avian physiological performance would be affected by MeHg and unpredictable food stress independently or combined. Contrary to my main prediction, combined exposure to MeHg and stress did not result in additive or synergistic effects. On the contrary, for several measures (BMR, fractional metabolic scope, moult

duration) birds exposed to both treatments had intermediate responses compared to birds exposed to one or the other. For other measures (MMR, feather quality and asymmetry) birds exposed to both treatments had responses equivalent to either or both of the stress-only and MeHg-only groups. This suggests no apparent combined effects of MeHg and unpredictable food stress, where each challenge affects the birds via different mechanisms but do not interact. According to prior research, body condition, metabolic rates, moult and feather quality should be influenced by each other and by energetic costs (Daan et al., 1990; Marmillot et al., 2016; Murphy et al., 1988; Portugal et al., 2007; Vézina et al., 2009), but my study demonstrates an independent effect of unpredictable food stress on body condition and metabolic rate whereas MeHg exposure primarily affected moult and feather quality.

3.4.1. Body condition

Body condition and change in body condition compared to pre-exposure did not show treatment effects during the exposure period. However, during the post-exposure period birds previously exposed to food stress had a greater increase in pre-migratory body condition compared to unstressed birds. Increases in body mass during post-breeding or pre-migratory periods were observed in other species (Gosler and Harper, 2000; King and Farner, 1963; Piersma et al., 2003). However, I cannot distinguish if this greater increase in body condition of food-stressed birds was due to a long-term effect of treatment on seasonal transitions, or due to the end of stress exposure independent of season. The removal of some birds at the end of the exposure period could also have influenced this result. Prior research on the effect of unpredictable food stress on body mass or condition has conflicting results. Some studies found no effect (Dall and Witter, 1998; Witter et al., 1995), while others observed an increase (Bednekoff and Krebs, 1995; Cornelius et al., 2017; Cuthill et al., 2000) or a decrease (Acquarone et al., 2002; Fokidis et al., 2012) in fat and body mass. In addition to species-specificity, birds' body mass adjustment strategies can also be season-specific (Witter et al., 1995). Furthermore, most research about food stress effects on body condition or mass measured body mass during exposure periods only. More studies on long-term effects after stress is removed are warranted.

Overall, my results indicate that previously stressed birds increased body condition for migration earlier than unstressed birds, which could consequently lead to an earlier departure for migration as observed in stop-over sites (Lupi et al., 2016; Schaub et al., 2008; Schmaljohann and Naef-Daenzer, 2011).

3.4.2. Metabolic rates

The results showed no effect of MeHg on BMR, MMR, or metabolic scope. This is in opposition to a prior study where zebra finches (*Taeniopygia guttata*) exposed to 0.6 $mg.kg^{-1}$ MeHg for 8 weeks showed an increase in BMR and a reduction in absolute metabolic scope compared to control individuals (Gerson et al., 2019). However, the authors found no significant effect of MeHg on MMR. In my study, the exposure dose was lower than the zebra finch study, and I measured metabolic rates during the 6-7th week of exposure instead of ≥ 8 weeks. However, my song sparrows' blood THg levels (5.25 ± 0.69 mean ± SD $mg.kg^{-1}$) at time of metabolic rate measurements were similar to the zebra finches reported levels (5.694 ±1.09 SD $mg.kg^{-1}$; Gerson et al., 2019). This suggests that my results differ either due to a difference in treatment duration or from a higher resistance of song sparrows to MeHg's deleterious effects on metabolic function. Species with different diets and habitats may differ in their capacity to cope with environmental MeHg. Song sparrows feed primarily on aquatic-emergent invertebrates during the breeding season and may differ from granivorous zebra finches. Further comparative studies on this topic are warranted.

In my study, birds in the stress-only treatment had lower BMR than control birds, which resulted in stressed birds having higher factorial metabolic scope than unstressed birds. Similarly, the absolute metabolic scope of Pekin ducklings was not affected by food restriction while factorial metabolic scope increased (Moe et al., 2005). Several studies observed BMR decreases in birds under food restriction or food unpredictability in adults birds (Liang et al., 2015; Mckechnie and Lovegrove, 1999; Noakes et al., 2013), suggesting that in times of food scarcity birds can adjust their energy expenditure. Such BMR adjustment can occur via a reduction of temperature (Doucette et al., 2012; Graf et

al., 1989; Hiebert, 1991; Moe et al., 2005), change in behavioural activity (Dall and Witter, 1998; Pravosudov and Grubb, 1997), or internal organs size reduction (Moe et al., 2004; Moe et al., 2005). Interestingly, BMR reduction due to unpredictable food stress did not carry-over into the post-exposure period, since the October measure showed no treatment effects. This finding is in agreement with prior studies where reduced BMR caused by food restriction during development returned to control level after the stress period ended (Liang et al., 2015; O'Connor et al., 2000; Zubair and Leeson, 1994). Such an energy conservation strategy during periods of hardship could allow birds to allocate more energy toward other energetic processes such as growth, moult, migration or reproduction (*compensation hypothesis*; Moe et al., 2004; Welcker et al., 2015). For example a reduced BMR could partially explain why stressed birds had a faster increase in body condition compared to unstressed individuals. Because BMR is associated with survival and reproductive fitness (Burton et al., 2011; Jimeno et al., 2020; Rønning et al., 2016), BMR adjustment has ecological consequences for the birds.

In song sparrows, food restriction during early development had no long-term effects on MMR (Schmidt et al., 2012). However, food restriction did reduce MMR in Pekin ducklings (*Anas platyrhyncos domesticus*) and European shag (*Phalacrocorax aristotelis*) nestlings (Moe et al., 2004; Moe et al., 2005). The lack of food stress on MMR in song sparrows thus appears consistent across ages, and differs from other species. More studies are needed to better understand what factors affect song sparrow MMR values, and how these may differ from other species.

3.4.3. Moult

Moult duration was strongly increased in birds exposed to MeHg, but not in those exposed to unpredictable food stress. The lack of effect from the unpredictable food stress treatment contradicts prior studies showing reduced feather growth rate (Andrews et al., 2021; Murphy et al., 1988; Swaddle and Witter, 1997b). Because the food stress treatment had effects on other measures, this suggests that song sparrows could compensate for unpredictable food stress on their moult. The observed MeHg effect

differs from a prior study where moult rate was positively correlated with blood THg levels at moult onset in startling (*Sturnus vulgaris;* Carlson et al., 2014). There are numerous methodological differences between this study and my experiment, including dose (0.19 vs. 0.75 and 1.5 $mg.kg^{-1}$ in the starling study) and duration of MeHg exposure (3 months in my study vs. 11 months). In addition, the starlings were exposed to MeHg during the full duration of their moult. Hence, I cannot determine if my differing results are caused by experimental difference or by the physiology of studied species. Starlings have longer moult (approximately 100 days; Carlson et al., 2014) than song-sparrows (approximately 68 days: 9.69 ±1.67 weeks mean ± SD across all individuals in my experiment) and these starlings may not be under the same migratory constraints as the song sparrows in this study, which could contribute to differing results. Further studies on moult speed under different challenging conditions are required. Since moult timing is correlated with migration timing (Cristol et al., 2014; Pulido and Coppack, 2004; Stutchbury et al., 2011), a delayed moult duration could affect migration departure. Alternatively, a moult-migration overlap such as observed in my study, could decrease the energy available for migratory behaviour (Podlaszczuk et al., 2016) and have consequences on individual survival (Hemborg and Lundberg, 1998; Nolan et al., 1992).

3.4.4. Feather quality

Contrary to prior studies (DesRochers et al., 2009a; Murphy et al., 1988; Pap et al., 2008), I did not detect an effect of unpredictable food stress on feather length, mass, or $mass.length^{-1}$ ratio. This suggests that the unpredictable food treatment was not associated with a strong stress response in song sparrows. However, my results did reveal an interaction between MeHg and primary feather $mass.length^{-1}$ ratio, and also feather length and mass. MeHg exposure resulted in increased $mass.length^{-1}$ ratio of P2, differing from the effect on other feathers. This suggests that only feathers growing during the exposure period were susceptible to deleterious MeHg effects. However, since P1 showed no strong effect of MeHg, more studies collecting multiples feathers are warranted. Prior studies documented MeHg effects on feather colour and mass (Evers et al., 2008; Giraudeau et al., 2015; White and Cristol, 2014). Since MeHg increased moult duration,

and longer moult is associated with heavier feathers (De La Hera et al., 2010), the effects of MeHg on feather quality could be an indirect outcome of the MeHg effect on moult duration. Ultimately, a negative effect of moult disruption on feather quality could have strong consequences for the birds (Echeverry-Galvis and Hau, 2013; Møller and Nielsen, 2018; Swaddle et al., 1996).

Interestingly, I observed an increase in mass.length^{-1} ratio from P1 to P9 feathers, also established in other species (Dawson, 2003; Dawson, 2005). Dawson (2005) suggested that the outer primary feather's greater mass per length could be beneficial for the bird either as a protection against abrasion or for an aerodynamic purpose. Nonetheless, MeHg effects on feather mass.length^{-1} ratio could increase fragility or risk of breaking over time.

I did not observe treatment effects on primary feather asymmetry. A prior study similarly did not find an effect of food deprivation on juvenile starling fluctuating asymmetry (Swaddle and Witter, 1997b), but an effect was observed in adult females (Swaddle and Witter, 1994). An increased flight feather asymmetry was also observed in individuals exposed to high MeHg levels (Evers et al., 2008). Most of the moult in my study occurred during the post-exposure period, and I did not observe any strong effects of MeHg (despite high blood THg levels at end of exposure) or stress on feather quality and asymmetry. This suggests that prior exposure to MeHg would not affect feather quality if birds move out of contaminated areas prior to the onset of their moult. Hence, pre-moult movement and reduced foraging during moult (Brown and Bryant, 1996; Portugal et al., 2010; Rimmer, 1988) could be part of positively selected behaviours under contamination exposure.

3.4.5. Conclusion

In summary, I measured the effects of unpredictable food stress and MeHg exposure on multiples measure of physiological performance associated with energy use. I observed that MeHg and stress appear to act through different physiological mechanisms with no combined effect on body condition, metabolic rate, moult, or feather quality. My results

suggest that wild songbirds may not be at risk of multiplicative effects from combined effects of MeHg and food stress on their energetic performance.

3.5. References

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Chapter 4

4. Effects of methylmercury and food stress on migratory activity in song sparrows[4]

4.1. Introduction

Migration is challenging and associated with high mortality rates in birds (Sillett and Holmes, 2002). In advance of both fall and spring migration, birds undergo preparatory hormonal, physiological and behavioural changes associated with flight, refueling, and navigation (Cornelius et al., 2013; Lupi et al., 2019; Ramenofsky and Wingfield, 2007). For instance, the thyroid hormone thyroxine (T4) is necessary for the expression of migratory preparation and flight (Pathak and Chandola, 1982; Pérez et al., 2016; Sharma et al., 2018). Similarly, corticosterone is associated with migratory departure (Eikenaar et al., 2014a; Eikenaar et al., 2020) and energy fueling during flight (Casagrande et al., 2020; Falsone et al., 2009).

During migration, birds cope with unpredictable stressors such as predators, inclement weather conditions, and variable resource availability that affect their behavior and physiology. For example, food deprivation increased migratory restlessness of captive birds (Biebach, 1985; Fusani et al., 2011; Gwinner et al., 1988) and food unpredictability increased non-migratory birds' baseline corticosterone and daily activity levels, and negatively affected their body composition (Eikenaar et al., 2014a; Fokidis et al., 2012). Environmental contaminants such as methylmercury (MeHg) can also increase birds' mortality risk during migration (Ma et al., 2018a; Roberts et al., 2014; Seewagen, 2020).

Mercury is a global environmental contaminant of concern, having persistent effects on many organisms' physiology and behavior (reviewed in Tan et al., 2009; Whitney and Cristol, 2017). Mercury is produced by both natural and anthropogenic processes such as fires, volcanoes, artisanal and small-scale gold production, coal combustion and

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production of non-ferrous metals (Dietz et al., 2009; Obrist et al., 2018; UNEP, 2013). Once deposited, inorganic mercury is converted to MeHg under anaerobic conditions, such as in aquatic sediments and floodplain soils (Bravo and Cosio, 2020; Regnell and Watras, 2019). MeHg is the most available and harmful form of mercury in the environment and biomagnifies up the food chain (Elliott and Griffiths, 1986; Hall et al., 2020; Newman et al., 2011). Terrestrial species, particularly songbirds with invertebrate diets, can bioaccumulate relatively high concentrations of MeHg (Ackerman et al., 2016a; Cristol et al., 2008). Documented impacts of MeHg on songbirds include adverse effects on endocrine and neural function, physiology, behavior, and reproduction (reviewed in Whitney and Cristol, 2017), but comparatively little research evaluates how MeHg exposure can affect migration.

Results of prior research suggested that MeHg can affect avian migration (reviewed in Seewagen 2020). MeHg exposure advanced birds' migratory departure (Seewagen et al., 2019), affected birds' metabolism (Gerson et al., 2019; Seewagen et al., 2022), reduced the energy available to use in take-off flight (Carlson et al., 2014), slowed homing flight in pigeons (Moye et al., 2016), and decreased warblers' ability to fly in a wind tunnel, suggesting impaired flight endurance (Ma et al., 2018b). MeHg exposure also affects endocrine systems in numerous species (reviewed in Tan et al., 2009). For example, MeHg exposure decreased developing birds' triiodothyronine (T3; Champoux et al., 2017; Wada et al., 2009) and impaired songbirds' acute stressor-induced corticosterone (Moore et al., 2014; Wada et al., 2009). However, there are conflicting findings on MeHg effects on songbirds' baseline corticosterone levels (Adams et al., 2009; Franceschini et al., 2009; Moore et al., 2014). MeHg exposure also negatively influenced body condition of migrant birds (Ackerman et al., 2019; Adams et al., 2020). Overall, the current evidence suggests that MeHg exposure could reduce survival during migration.

Although birds often experience contaminants and other environmental stressors concurrently in the wild, no study to date has investigated how simultaneous exposure to MeHg and food stress (unpredictability of food availability) could affect migration. Importantly, past studies indicate that both elevated corticosterone levels (Koren et al., 2012; Latta et al., 2016; Schultner et al., 2014) and exposure to MeHg (Heddle et al.,

2020; Lavoie et al., 2014b; Ma et al., 2018a; Paris et al., 2018) can have carry-over effects. However, the physiological mechanisms of the carry-over effects of MeHg exposure remain uncertain. Hence, my objective was to determine if summer exposure to dietary MeHg and/or food stress could affect fall migratory behavior in song sparrows, *Melospiza melodia*, using nocturnal activity as a proxy for fall migratory disposition. My hypotheses were that 1) MeHg exposure and/or unpredictable food stress have carry-over effects on migratory nocturnal activity, 2) hormone levels (corticosterone, T4) and body condition are mediators of migratory nocturnal activity and 3) these nocturnal activity mediators can be affected by MeHg and/or food stress exposure.

Because the treatment exposures could have both direct and indirect carry-over effects on migratory activity, I had five predictions. I first predicted that unpredictability of food availability would directly increase song sparrows' migratory activity, similar to prior studies (Biebach, 1985; Fusani et al., 2011; Gwinner et al., 1988). Second, I predicted that MeHg would positively or negatively affect migratory behavior either by inducing hyperactivity (Seewagen et al., 2019; Swaddle et al., 2017) or by reducing energy availability for activity (Carlson et al., 2014; Gerson et al., 2019), respectively. Third, I predicted that combined exposure to MeHg and food unpredictability could have either additive or compensatory interactive effects on migratory activity depending if MeHg positively or negatively affects individual activity levels. Fourth, I predicted an increase of mediators of migratory activity (T4, corticosterone, body condition) from pre-migratory to migratory periods, and a significant positive correlation between nocturnal activity and these mediators. Finally, I also predicted that treatment exposure would disrupt these mediators, as shown in prior studies (Ackerman et al., 2019; Adams et al., 2020; Dickens and Romero, 2013; Fokidis et al., 2012; Tan et al., 2009) and that such disruptions could change with time, for example, due to seasonal changes or the cessation of experimental exposure to MeHg and food stressors.

4.2. Methods

4.2.1. Bird capture and housing

Species capture, housing and experimental design with treatment exposure was described in chapters 2 and 3. Briefly, from April 2018, 49 adult song sparrows were housed at the University of Western Ontario's Advanced Facility for Avian Research (AFAR) in London, Ontario (Animal use protocol # 2017-161). The light schedule was adjusted each week to follow the local natural photoperiod, to maintain normal migratory schedules. From 15-16 May to 12-13 August 2018 (90 days), I exposed birds to unpredictable food stress and-or environmentally relevant doses of MeHg, as described in chapter 3. After exposure ended, 14 birds (2-5 per treatment) were euthanized so that 32 birds (8 per treatment: 5-8 males, 0-3 females) were monitored during a post-exposure period until they were euthanized on 31 October to 4 November 2018. During the post-exposure period the birds were given control diet up to the end of the experiment. The study timeline is illustrated in Figure 4.1. THg analyzes of blood and agar food were performed at the Biotron at the University of Western Ontario, as described in chapter 2.

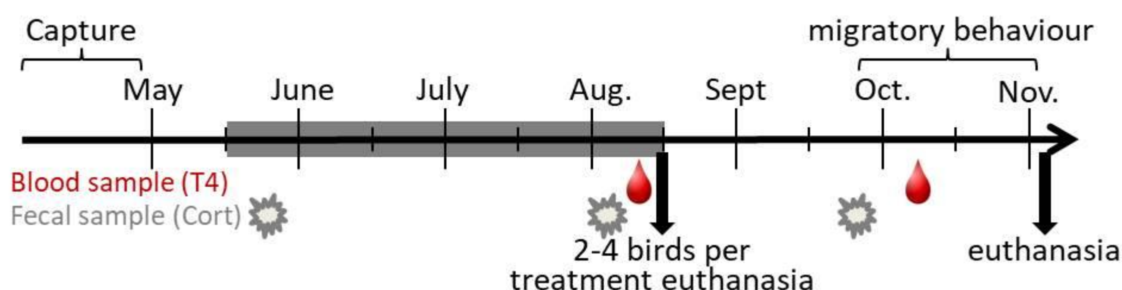

Figure 4.1: Timeline of this study. Grey highlighted area indicates the period of treatments exposure. The red drops indicate the week of blood sampling for thyroxine measures, while the ligh grey patches indicate the days of fecal sample collection for fecal corticosterone metabolites.

4.2.2. Migratory restlessness behavioural analysis

Birds' nocturnal activity was monitored and recorded from 25 September to 1 November 2018, by my collaborator RW. Prior studies indicate that the intensity and persistence of migratory restlessness positively correlates to the likelihood and duration of a bird migrating in the wild (Berthold, 1973; Bulte et al., 2017; Eikenaar et al., 2014b). Birds were recorded from one hour before lights off to one hour after lights on (range of approximately 14-15.5 h per bird over the duration of the recording period). Eight infrared (IR) emitters and eight IR-sensitive video cameras were set up to each record the nocturnal behavior of four birds. The cameras connected to a computer running Noldus EthoVision® behavioural analysis software (version 10.0.826, Noldus Information Technology).

Videos were recorded and saved for analysis following the completion of the recording period. Video analyzes were completed by my collaborator (Rebecca Whiley). There were 36 nights of recordings per bird, with five exceptions due to technical errors and the death of one bird part way through the study. Videos were divided into 15-min segments for analysis. Detection settings were optimized using static subtraction of a unique background image with subjects digitally removed that was collected each night. The detection settings ranged from 9-245 for dark contrast, 2-575 for subject size, 6-7 for activity threshold, 2-3 for background noise filter, and had a sample rate of $1.4985.s^{-1}$, low video pixel smoothing, track noise reduction off, compression artifacts filter on, and contour erosion and dilation of 1. Distance units were calibrated for each video using the calibrate scale in the software.

The Noldus EthoVision software extracted three movement variables (distance moved [cm], time moving [s], and time mobile [s]) by assessing changes in a bird's center-point (center of body detected against a light background) and body contour (change in body shape) (Kelly et al., 2020). Distance moved and time moving were determined from movement of the bird's centre point, while time mobile was determined from changes in the bird's body contour. Birds were defined as *moving* when their centre point movements were ≥ 2 $cm.s^{-1}$ and *mobile* when their total body area changed by ≥ 20 % (user-defined thresholds). Migratory restlessness behaviors like short flights, hopping, and jumping

were detected by centre point and body contour movement tracking, while stationary motions like wing whirring were detected only by body contour movement tracking. Direct observation of these videos confirmed that nocturnal activity of song sparrows consisted almost exclusively of hopping and short flights around the cage, and/or wing fluttering. In non-migratory condition, song sparrows sleep throughout the night and have almost no detectable activity. I thus interpret the nocturnal activity that was recorded as a good proxy for migratory tendency or motivation.

4.2.3. Blood collection and T4 analyzes

Birds were staggered in two groups spread among sex and treatment, so that the second group was blood-sampled 24 h after the first group. I took an initial blood sample from all birds on 1-2 May 2018, before the experiment started, in order to confirm the birds' low initial mercury levels. Then starting on 16-17 May 2018, I took a blood sample once every 4 weeks. To collect blood samples, a group of researchers entered the room and punctured the birds' wing vein with a 26-gauge needle and collected 50-200 µL of blood into heparinized microhematocrit tubes. Samples were collected within 20 min of the group entering the room. If samples could not be centrifuged quickly, they were kept on ice or in a refrigerator. Within 5-90 min of collection, I transferred 25-50 µL of whole blood into microcentrifuge tubes for later THg analysis, while the rest was centrifuged to separate plasma from red blood cells. Each sample was then stored at -80 °C until analyzes. Blood samples for T4 analyzes were collected on 8-9 August and 3-4 October 2018.

T4 levels were assessed via an ELISA kit (Arbor Assays, # K050-H5), following the kit protocol for a 100 µL sample format. Hormone assays were validated for song sparrow plasma by using parallelism and accuracy tests. Due to a technical problem, one plate did not produce usable results, reducing my sample size, with 32 samples lost. The 43 usable samples left were balanced within treatment and month (4-5 birds per treatment in each month) but only 11 of the 32 individuals had samples for both August and October. Among the usable samples, four had an absorbance value outside of the standard curve;

for statistical analyzes, these undetectable samples were set to the concentration of the lowest detected sample on their plate. The inter-assay coefficient of variation was determined from homogenized plasma pool samples analyzed on each plate. The intra-assay coefficient of variation was 15.1 % and the inter-assay coefficient of variation was 8.6 %.

4.2.4. Fecal collection and corticosterone metabolite analyzes

Fecal corticosterone was measured to provide a time-integrated measure of glucocorticoids in circulation (Goymann et al., 2002) and reduce the number of blood collections per bird. I collected overnight fecal droppings every two weeks following Eikenaar et al., (2014a). On sampling days, clean paper sheets were placed on the bottom of each bird's cage beginning at 3 pm and were collected on the next morning at 10 am. Placing and removing the paper took approximately 30 min total, and should have induced minimal disturbance to the birds. In the morning, once the paper was collected, the fecal droppings were placed into a microcentrifuge tube before being stored at -80 °C until analyzes. I analyzed fecal corticosterone metabolites of samples collected on 26 May (11 days since start of treatments, exposure), 4 August (81 days, exposure) and 29 September (137 days, post-exposure). To extract steroids and steroid metabolites from the fecal samples, I thawed the samples and dried them at 90 °C for 1 h to destroy bacterial enzymes (following Turriani et al., 2016). Once dry, I placed each sample into a glass tube and manually ground it with three glass probes for 60 s. I then weighed the samples (0.2162 ± 0.0180 g mean ± S.D.) and added 60 % methanol (as validated by my pilot tests and also recommended in Palme et al., 2005) in proportion of the dry mass (1 mL methanol per 0.1 g fecal matter). I then vortexed the sample for 1 min with hand-vortex and 30 min on plate shaker at 700 rpm before centrifugation for 30 min at 1,644 g. Following metabolite extraction, I placed 1 mL of the supernatant into a new microcentrifuge tube which was then kept at -20 °C until analyzes. The extraction protocol recovery percentage was tested by adding a 25 μL of corticosterone standard from the ELISA kit into 475 μL of 60 % methanol tube before following the same extraction protocol and sample analysis. I obtained a recovery of 115.92 ± 27.22 %.

I analyzed the fecal corticosterone metabolite concentration using a corticosterone ELISA kit (Arbor Assays ELISA #K014-H) following the kit protocol for 100 µL format. The intra-assay coefficient of variation was 6.55 %. The inter-assay coefficient of variation was determined from 2 samples analyzed on each of the 4 plates and was 10.7 %. I expressed fecal corticosterone data in $pg.mL^{-1}$ representing the concentration in corticosterone metabolites in the methanol supernatant that is then used as an estimate for the dry fecal concentration.

4.2.5. Body condition

Methods for body condition measurement are described in chapter 3. Briefly, I measured each bird's tarsus length to the nearest 0.1 mm using dial calipers. Bird body mass was measured with an electronic balance to the nearest 0.01 g once every two weeks from the beginning to the end of the experiment. Here I include mass data measured during the migratory recording period only, with mass measured on 1 October, 15 October, 29 October. I calculated body condition as the mass relative to an individual's size as body mass (g) divided by tarsus length (mm) and then standardized it as a z-score (with mean = 0 and SD = 1) for each individual (Ackerman et al., 2019). This resulted in a continuous variable with higher values corresponding to a bird with higher mass relative to its size.

4.2.6. Data analysis

All statistical analyzes were conducted in R Version 4.0.3 (R Core Team, 2020). All results are presented as means ± SE, and level of significance set at $P < 0.05$. Assumptions of residuals normality and homogeneity were validated before interpreting the results.

4.2.6.1. Nocturnal activity data preparation

For the nocturnal activity data collected by EthoVision software, I first filtered data to include only data collected during night (lights off period). Data from one night of recording for 4 individuals were lost due to a camera failure. To estimate migratory behaviour, I created a binary score of activity for each 15 min of video recording. If all three of the movement variables extracted by EthoVision (distance moved, time moving, and time mobile) were equal to zero (no movement detected) the bird was considered inactive and assigned a score of 0. If any movement was detected during the 15 min, the bird was considered active and assigned a score of 1. During sampling periods, some birds would not be detected by the software. Because these cases of non-detection mostly involved birds not moving, I assigned any cases where a bird was undetected for over 50 % of the 15 min period a score of 0. This occurred for 298 out of 56,502 time bins. Omitting this correction led to qualitatively identical results.

For each individual and for each night, I then calculated the proportion of 15-min intervals in which the bird was active over the night (sum of all the scores for each night, divided by the number of intervals recorded). This yielded a continuous variable ranging from 0 to 1, representing the proportion of the night that birds were active, hence nocturnal activity duration within a night.

4.2.6.2. Nocturnal activity data analysis

I assessed treatment group differences in nocturnal activity duration by constructing a linear mixed effects model (lme) using the *lmerTest* package (Kuznetsova et al., 2017). I first centered the variable *night* by dividing each night's numerical value by the mean of all unique values of nights. Centering a continuous variable does not affect the relationship (slope) between the bird nocturnal activity and night but changes the intercept to obtain more biologically relevant estimates. It also had the advantage of removing a collinearity issue associated with interactions of fixed effects in some of the models tested. The model's fixed effect variables included mercury, food stress, and night and their triple interaction. The model's random effects include two random intercept

effects for bird ID and centered night, to account for both repeated measures and the high variability from one night to the other. Hence, the model includes the night variable both as a fixed effect and as a random effect. The fixed effect allowed me to assess behavioural variation across nights, while the random specification accounted for the repeated measure while keeping each night independent from one another. This way, the daily changes in handling procedures or environmental factors that make each night unique are accounted for independently in the statistical model. Bird sex was not included in the model since preliminary checks indicated no influence of the variable on nocturnal activity. Model results were obtained by fitting the model with restricted maximum likelihood, and each model R^2 was extracted via the *MuMIn* package (Barton, 2020).

In order to better understand the three-way interactions in the model above, I split data based on food stress treatment, and then ran two similar models to re-analyze the data for stressed and unstressed birds separately. When required, fixed effect removal was validated by likelihood ratio test via the *drop1* function from the *stats* package (R Core Team, 2020). The *drop1* function compares the AIC of several models in which one fixed variable or interaction was removed. The function output will give a p-value indicating if the variable removal significantly improved the model AIC or not. I present the result of the final selected model fitted with restricted maximum likelihood.

4.2.6.3. Mediators of migratory behaviour data analysis

Following analysis of treatment effects on nocturnal activity, I evaluated the effects of treatment and seasonality on mediators of migratory behaviour (T4, fecal corticosterone metabolites, and body mass) and I assessed the relationship between these mediators and nocturnal activity. To do this, I conducted two separate models for each of the mediators. To test the effect of treatment and date on T4 hormone concentration, I performed a linear model (lm) on log-transformed blood T4 concentrations as the dependent variable and mercury, stress, sampling date and their 3-way interaction as fixed effects. Because most birds in my limited sample size had only one data point, I did not include bird ID as a random variable to reduce the issue of model singularity. The model results were not

affected by this modification. I then used likelihood ratio test via the *drop1* function to identify significant variables of a final model. Then to assess the relationship between nocturnal activity duration and T4 concentration, I used a subset of the nocturnal activity data, including only the nights prior to blood sample collection. I created a lm including nocturnal activity duration as the response variable and untransformed T4 levels, MeHg treatment, stress treatment, and their interactions as independent variables. This saturated model was then reduced to the most influencing variables via likelihood ratio test using the *drop1* function.

To evaluate treatment effects on fecal corticosterone metabolite concentrations, I ran a lme model on log-transformed corticosterone values from the full dataset available (n = 48 in May sample; one sample point was removed). As a result robustness check, I also tested the same model on a dataset comprising only the birds kept until November (n = 32). The model fixed effects included stress and mercury treatments, sampling date, and their 3-way interaction, as well as the bird tarsus size centered on their mean and the bird's blood sampling group as covariates since a preliminary data check showed an influence of these variables. The bird ID was included as a random variable to account for repeated measures. I removed one bird from the dataset to allow for a better model fit due to its outlier value (determined by model validation tests). This bird was sampled only once in May due to its unexpected death during handling. I then did a Tukey multi-comparison post-hoc test via the *glht* function from the *multcomp* package (Hothorn et al., 2008) to determine how fecal corticosterone metabolites, varied across groups and over sampling dates. Then to assess the relationship between nocturnal activity and fecal corticosterone metabolites, I used a subset of the nocturnal activity data for the nights prior to fecal sample collection. I created a lm including nocturnal activity duration as the response variable and log-transformed fecal corticosterone metabolites, MeHg and stress treatments, and their interactions as independent variables. This saturated model was then reduced to the most influencing variables via likelihood ratio test using the *drop1* function.

Finally, I assessed if body condition influenced nocturnal activity duration, I used the nocturnal activity data of the night prior to mass measurement. I created a lme model with

nocturnal activity as the dependent variable and with mercury, stress, body condition, measurement date and their 4-way interaction as fixed effects. Bird ID was included as a random effect. I used likelihood ratio tests via the *drop1* function to identify significant variables of a final model. A Tukey multi-comparison post-hoc test was used to compare the effects of treatment and date on nocturnal activity.

4.3. Results

4.3.1. Nocturnal activity

I first assessed the carry-over effects of food stress and MeHg exposure treatments on overnight nocturnal activity duration. The 3-way interaction effect between night × mercury × stress treatments was significant, along with the interaction of stress × night (Table 4.1a; Figure 4.2). These interactions indicate that the effects of the food stress manipulation changed over time (nights) and this nights × stress interaction varied with MeHg exposure. The model was significantly better than a null model (Table 4.1b). In the final model, the mercury main effect had the highest parameter estimate of all the fixed effects. However, as a main effect it was not statistically significant ($p = 0.057$), likely due to interactions with other factors. I still interpret the effect of mercury as biologically important given the high parameter estimates and the statistically significant interaction. Mercury exposed birds appeared to have higher nocturnal activity throughout the experiment compared to uncontaminated birds (Figure 4.2). I also did not find a main effect of food stress (Table 4.1a).

Table 4.1: Results of song sparrow's nocturnal activity final models. Final model's significant variables ($p<0.05$) are displayed in bold and italic.
a. Parameter estimates of final lme models predicting treatment and date effects on song sparrow nocturnal activity duration. Indication of [y] signal which factor group of the data (e.g., group exposed to mercury) is compared to the reference group (e.g., unexposed birds). Marginal R^2 indicates the variance explained only by fixed effects and conditional R^2 indicates the variance explained by the fixed and random effects.
b. ANOVA comparison between the null model and the final model predicting song sparrow nocturnal activity duration.

a. Nocturnal activity main model				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	0.32	0.044	7.21	**<0.001**
stress [y]	0.016	0.059	0.27	0.79
MeHg [y]	0.12	0.059	1.98	0.057
night	0.000	0.002	0.040	0.97
stress [y] × MeHg [y]	-0.079	0.083	-0.95	0.35
stress [y] × night	***0.005***	***0.001***	***4.84***	***<0.001***
MeHg [y] × night	0.001	0.001	0.71	0.48
stress [y] × MeHg [y] × night	***-0.007***	***0.001***	***-5.19***	***<0.001***
Random Effects	*Variance*	*Std.Dev.*	*N*	
Bird.ID τ_{00}	0.013	0.12	32	
night τ_{00}	0.008	0.089	37	
Residuals σ^2	0.015	0.12		
Intraclass-correlation coef. (ICC)	0.59			
Observations	1146			
Marginal R^2 / Conditional R^2	0.070 / 0.62			

b. ANOVA comparison between null model and final model				
	npar	*AIC*	*X^2 (Df)*	*p-value*
Null model	4	-1304.3		
Final model	11	-1341.7	51.31 (7)	< 0.001

To better understand the above 3-way interaction, I ran two follow-up models after separating the dataset by stress treatment. For the food-stressed birds (birds from stress-only treatment and coexposure to MeHg and food stress), there was a significant main effect of night, and a significant night × mercury interaction (Figure 4.2; Table 4.2a). For the birds not exposed to food stress (birds from control treatment and MeHg-only exposure), there was no significant change in migration duration over time, nor a significant effect of MeHg (Table 4.2b).

Table 4.2: Results of song sparrow's nocturnal activity final models on dataset divided by stress. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

a. Parameter estimates of final models predicting MeHg treatment effects on nocturnal activity duration in subset dataset only including food stressed song sparrows (stress-only vs coexposure to MeHg and stress treatment).

b. Parameter estimates of final models predicting MeHg treatment effects on nocturnal activity duration in subset dataset only including non-food stressed song sparrows (control vs MeHg-only treatment).

a. Model on stressed birds				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	0.33	0.041	8.194	**<0.001**
MeHg [y]	0.038	0.053	0.720	0.48
night	***0.005***	***0.002***	***2.81***	***0.007***
MeHg [y] × night	***-0.006***	***0.001***	***-6.39***	***<0.001***
Random Effects	*Variance*	*Std.Dev.*	*N*	
Bird.ID τ_{00}	0.011	0.10	16	
night τ_{00}	0.0093	0.097	37	
Residuals σ^2	0.016	0.13		
Intraclass-correlation coef. (ICC)	0.56			
Observations	*572*			
Marginal R^2 / Conditional R^2	0.047 / 0.580			

b. Model on non-stressed birds				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	0.319	0.047	6.74	**<0.001**
MeHg [y]	0.117	0.064	1.82	0.091
Random Effects	*Variance*	*Std.Dev.*	*N*	
Bird.ID τ_{00}	0.016	0.13	16	
night τ_{00}	0.0062	0.079	37	
Residuals σ^2	0.014	0.12		
Intraclass-correlation coef. (ICC)	0.62			
Observations	574			
Marginal R^2 / Conditional R^2	0.086 / 0.65			

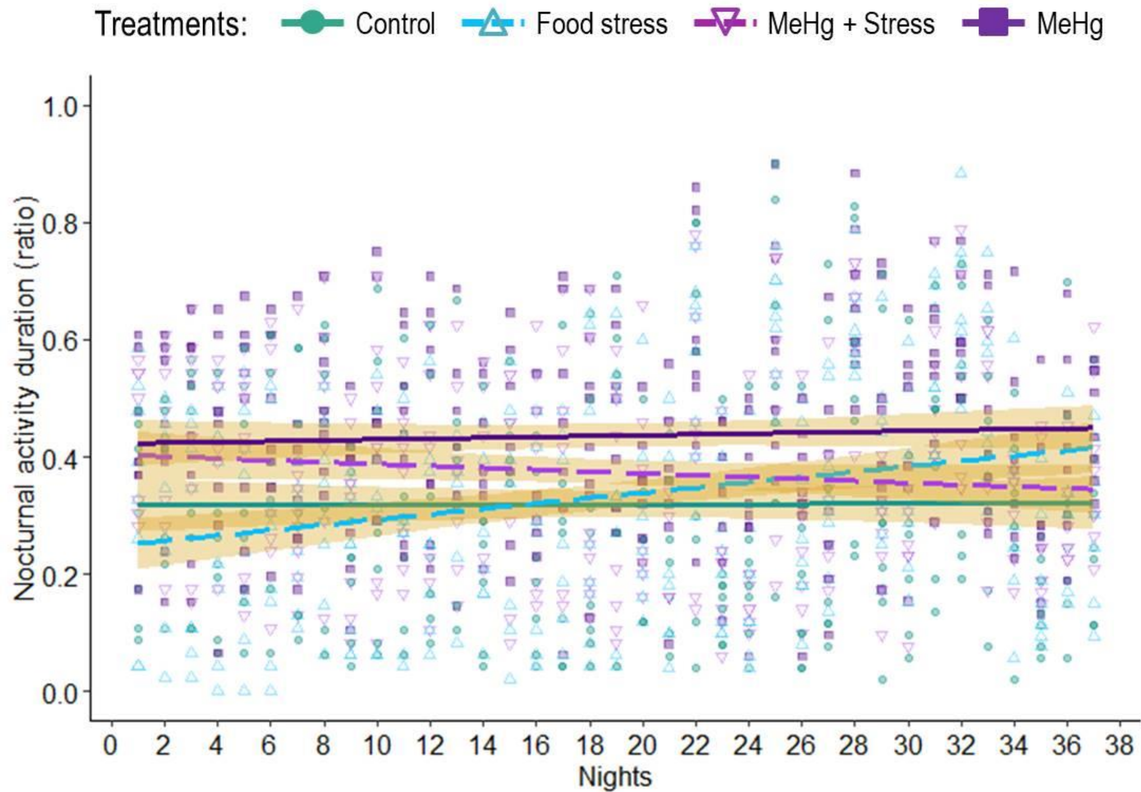

Figure 4.2: Nocturnal activity duration ratio (mean ± SE) over nights of recording from 25 September (night 1) to 1 November (night 38). A nocturnal activity ratio of 1 would indicate that birds are active all night long, while a ratio of 0 indicates birds not moving during the night. Lines were fitted via linear model method. Colour of symbols and lines indicate the bird's treatment group of control (dark blue line and circle, n = 8), stress (dashed light blue line and triangle, n = 8), MeHg exposure (dark purple line and square, n = 8), or coexposure to MeHg and stress (dashed light purple line and inversed triangle, n = 8).

4.3.2. Hormones and body condition

Next, I assessed if known mediators of migratory behaviour (T4, corticosterone, body condition) were affected by seasonality or experimental treatments and if they correlated with nocturnal activity. Plasma concentration of T4 was not affected by treatments or sampling date since the null model was selected by the likelihood ratio test

(Table 4.3a; Figure 4.3a). Mean T4 hormone concentrations were 832.07 ± 87.01 $ng.mL^{-1}$ (mean ± SE) in August and 979.67 ± 108.58 $ng.mL^{-1}$ in October. Nocturnal activity on the night prior to blood sampling was not related to morning plasma T4 concentration since the variable was not selected in the final model (Table 4.3b). However, birds exposed to MeHg had higher nocturnal activity on the night prior to blood sampling (Table 4.3b; Figure 4.3b).

Table 4.3: Result of plasma T4 concentration final models, selected after variable removal via *drop1* function.
a) Parameter estimates of final linear models predicting treatment effects and sampling date on song sparrow plasma T4 concentration. Null model was selected.
b) Parameter estimates of final linear models predicting relationship between T4 and nocturnal activity. T4 concentration variable do not appear since it was removed during the model selection steps. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

a. Plasma T4 concentration final model on treatment effects				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	6.71	0.07	101.069	**<0.001**
Observations	43			
Multiple R^2 / Adjusted R^2	0.000 / 0.000			

b. Final linear model between T4 and nocturnal activity.				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	0.310	0.052	6.0	**<0.001**
MeHg [y]	***0.17***	***0.070***	***2.41***	***0.026***
Observations	22			
Residual standard error	0.16 on 20 degrees of freedom			
Multiple R^2 / R^2 adjusted	0.225 / 0.186			
F-statistic:	5.796 on 1 and 20 degrees of freedom			
Model p-value	0.026			

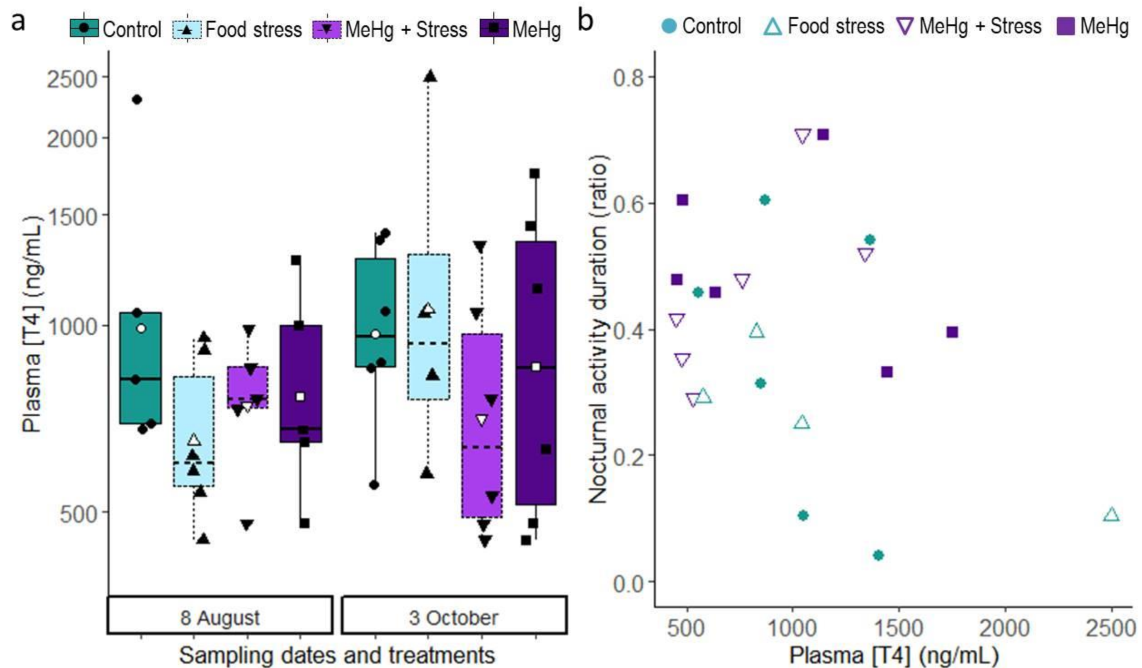

Figure 4.3: Thyroxine (T4) hormones levels ($ng.mL^{-1}$). a) Seasonal variation of 8-9 August (n = 21) versus 3-4 October (n = 22) levels. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: control (dark blue with circles, August n = 5, October n = 6), food stress (light blue with triangles, August n = 6, October n = 4), combined MeHg and food stress (light purple with inverted triangles, August n = 5, October n = 6), MeHg (dark purple with squares, August n = 5, October n = 6). The white symbols indicate the mean hormone level for each treatment group and time point. b) Absence of relationship between nocturnal activity duration on the night before blood sampling and plasma T4 concentration. Symbols indicate the treatment groups of control (light blue dot, n = 6), stress (blue triangle, n = 4), MeHg exposure (green inverted triangle, n = 6), or coexposure to MeHg and stress (dark green square, n = 6).

Analysis of fecal corticosterone metabolite levels revealed a significant 3-way interaction of stress × MeHg × sampling date (Table 4.4a; Figure 4.4a). Hence, treatments differentially affected the hormone, and the outcome was dependent on the month of sample collection. Post-hoc analysis indicated that, overall, there was no difference between treated birds and control birds in May and August, but birds exposed to

combined MeHg and stress had higher fecal corticosterone metabolites than control birds in September (Figure 4.4a). Contrary to other treatments, control birds decreased their fecal corticosterone metabolite concentrations from May to August. However, other treatment groups had monthly differences when compared to September. Indeed, fecal corticosterone metabolite levels of stress-only birds increased from May to September, while MeHg-only birds increased from August to September and birds exposed to both MeHg and stress had higher fecal corticosterone metabolite concentrations in September compared to the two previous sampling dates (Figure 4.4a). The final model also contained the main effect of stress, the main effect of date, and the interactions of stress × date and MeHg × stress were also significant (Table 4.4a). This indicates that stressed birds had overall lower fecal corticosterone metabolites than unstressed birds throughout all measurement time points, and that stress effects change with time. Also, combined exposure to MeHg and stress resulted in an overall increase in corticosterone, different than exposure to MeHg or stress alone. The main effect of date resulted from the decrease in fecal corticosterone metabolite levels in August (169.30 ± 8.267 pg.mL^{-1}) compared to May (197.45 ± 10.89) or September (281.39 ± 19.92). Fecal corticosterone metabolites were also related to the bird's tarsus size and blood sampling group (Table 4.4a). Individuals in the second blood sampling group had higher fecal corticosterone across the three sampling time points, and fecal corticosterone metabolites decreased in birds with longer tarsus length.

I also examined if glucocorticoids were linked to migratory activity. Nocturnal activity on the night prior to fecal sample collection was positively related to fecal corticosterone metabolites and mercury treatment (Table 4.4b). Birds with higher corticosterone and those exposed to MeHg had greater nocturnal activity duration on the night before the fecal sample collection (Figure 4.4b).

Table 4.4: Result of fecal corticosterone metabolite concentration final models, selected after variable removal. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

a) Parameter estimates of final lme models predicting treatment and date effects on song sparrow fecal corticosterone metabolite concentration. Indication of [y] and [month] indicates which factor group of the data is compared to the reference group (e.g., unexposed group or month of May).

b) Parameter estimates of final linear models predicting the relationship between fecal corticosterone metabolite and nocturnal activity.

a. Fecal corticosterone metabolite final model on treatment effects				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	6.59	0.12	56.98	**<0.001**
MeHg [y]	-0.11	0.13	-0.89	0.38
stress [y]	***-0.30***	***0.13***	***-2.40***	***0.018***
date [Aug.]	***-0.40***	***0.13***	***-3.18***	***0.002***
date [Sept.]	0.036	0.14	0.26	0.80
tarsus	***-0.12***	***0.041***	***-2.95***	***0.005***
group	***0.21***	***0.057***	***3.72***	***0.001***
MeHg [y] × stress [y]	***0.50***	***0.18***	***2.81***	***0.006***
MeHg [y] × date [Aug.]	0.28	0.18	1.52	0.13
MeHg [y] × date [Sept.]	0.33	0.20	1.64	0.11
stress [y] × date [Aug.]	***0.60***	***0.18***	***3.38***	***0.001***
stress [y] × date [Sept.]	***0.53***	***0.20***	***2.70***	***0.008***
MeHg [y] × stress [y] × date [Aug.]	***-0.69***	***0.25***	***-2.74***	***0.008***
MeHg [y] × stress [y] × date [Sept.]	-0.46	0.28	-1.63	0.11
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	0.00099	0.032	48	
Residuals σ^2	0.084	0.29		
Intraclass-correlation coef. (ICC)	0.03			
Observations	126			
Marginal R^2 / Conditional R^2	0.45/ 0.46			
b. Final linear model between fecal corticosterone metabolite and nocturnal activity.				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	-1.22	0.52	-2.34	**0.26**
MeHg [y]	***0.13***	***0.054***	***2.34***	***0.026***
Log(corticosterone)	***0.21***	***0.073***	***2.87***	***0.008***
Observations	32			
Residual standard error	0.15 on 29 degrees of freedom			
Multiple R^2 / R^2 adjusted	0.38 / 0.34			
F-statistic:	8.86 on 2 and 29 degrees of freedom			
Model p-value	0.00099			

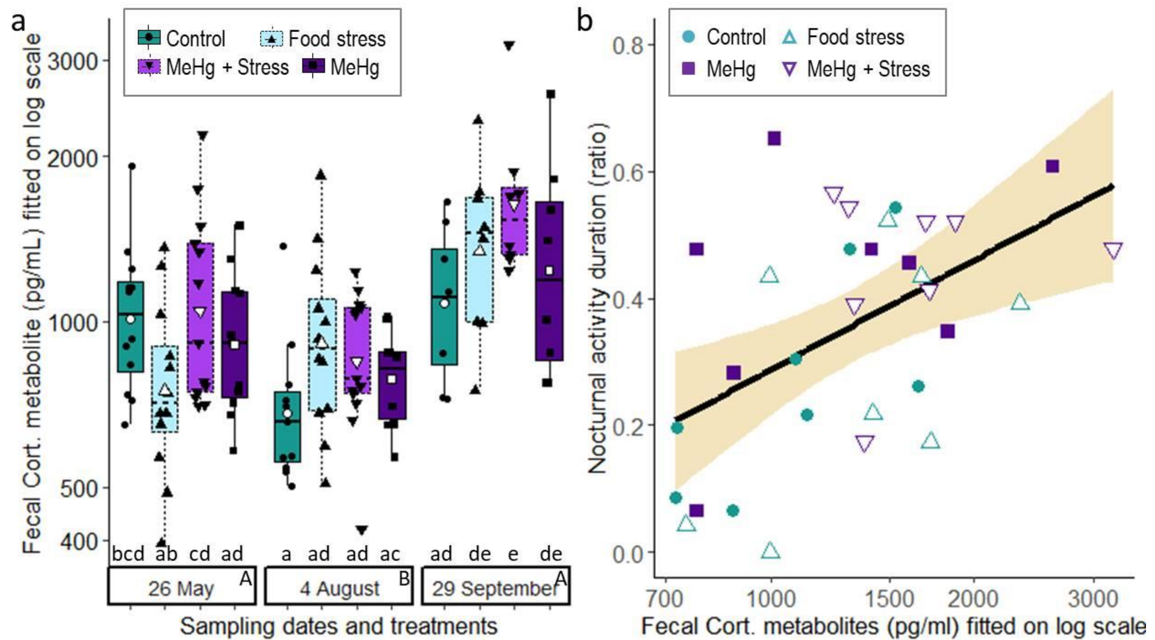

Figure 4.4: Fecal corticosterone metabolite levels (pg.mL^{-1}). a) Seasonal variation of log-transformed values across treatments between 26 May (n = 48), 4 August (n = 46) versus 29 September (n = 32). Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. Boxplots colour and symbols indicate treatment groups: control (dark blue with circles), food stress (light blue with triangles), combined MeHg and food stress (light purple with inverted triangles), MeHg (dark purple with squares). The white symbols indicate the mean hormone level for each treatment group and time point. Lower case letters indicate significant difference between treatments for all sampling dates (Tukey post-hoc test $p < 0.05$) while upper case letters indicate significant difference between months (Tukey post-hoc test $p < 0.05$). b) Result of linear model between nocturnal activity duration on the night before fecal collection and fecal corticosterone metabolite concentration. Symbols indicate the birds treatment group of control (blue circles, n = 8), stress (blue triangles, n = 8), MeHg exposure (purple inverted triangles, n = 8), or coexposure to MeHg and stress (purple squares, n = 8).

Finally, the results of food stress effects on body-condition throughout the experiment were described in chapter 3 and will not be repeated here. For this chapter, I checked if body condition influenced migratory activity. Standardized body condition was not

associated with nocturnal activity duration since the variable was not retained in the final model ($p > 0.1$; Table 4.5b). Across the three measurement time points, nocturnal activity was influenced by the interaction of stress × night (Table 4.5b). There was no difference in nocturnal activity duration between stressed and unstressed treatment groups for each night prior to body condition measurement (Tukey post-hoc $p > 0.1$). However, birds in the two food-stressed treatment groups had lower nocturnal activity during the two first measurements compared to the last one (Tukey post-hoc $p < 0.05$), but unstressed birds did not.

Table 4.5: Parameter estimates of final linear models predicting relationship between body condition and nocturnal activity. Body condition did not appear since it was removed during the model selection steps. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

Final linear model between body condition and nocturnal activity.				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	0.41	0.047	8.69	**<0.001**
stress [y]	-0.076	0.066	-1.15	0.25
night [15 Oct.]	0.023	0.053	0.44	0.66
night [*29 Oct.*]	0.000	0.053	0.003	0.99
stress [y] × night [15 Oct.]	-0.020	0.075	-0.27	0.79
stress [y] × night [29 Oct.]	***0.16***	***0.075***	***2.17***	***0.034***
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	0.012	0.11	32	
Residuals σ^2	0.023	0.15		
Intraclass-correlation coef. (ICC)	0.35			
Observations	96			
Marginal R^2 / Conditional R^2	0.085 / 0.40			

4.4. Discussion

Confirming my first hypothesis, spring-summer exposure to food stress alone or in combination with environmentally relevant MeHg doses caused carry-over effects on the migratory behaviour of song sparrows. However, birds' nocturnal activity changed over time in different ways depending on their previous treatment. The treatments' carry-over effects persisted for at least six weeks following the end of treatment exposure, an effect that has not been documented previously. Among the three hypothesized mediators of

migratory behaviour measured, two were influenced by treatment exposure, but only fecal corticosterone was positively correlated to nocturnal activity. To my knowledge, this is the first time that combined exposure to MeHg and stress has been shown to have additive carry-over effects on corticosterone in songbirds and that both corticosterone and MeHg exposure may influence nocturnal activity. These results suggest that songbirds breeding at MeHg-contaminated sites or in stressful habitats might have altered behaviour during fall migration, potentially via indirect effect on corticosterone levels.

A growing number of studies document the potential for carry-over effects of stressors (Koren et al., 2012; Latta et al., 2016), including MeHg exposure (Heddle et al., 2020; Ma et al., 2018a). The main proposed mechanisms for such carry-over effects involve long-term energetic trade-offs (Harrison et al., 2011) or trait selection during developmental life-stages that affect later life-stages (Moore and Martin, 2019). My results suggest that the mechanisms behind carry-over effects may be subtle because treatments did not influence the birds' hormone levels during the exposure period. My study shows that behavioural disruption could be experienced during fall migration, several months after exposure to MeHg and food stress ended, and despite low blood mercury concentrations during this period. Indeed, exposed birds' change in nocturnal activity was probably independent of their blood MeHg concentration at the time of migratory behaviour because they had low blood THg levels at that time. Also, both MeHg-only and co-exposure treatment birds had similar blood total mercury throughout the experiment (chapter 2). Alternatively, the results may suggest MeHg effects on migratory activity occur with very low blood levels, but this possibility seems unlikely. The carry-over effects on behaviour and physiology observed in my study may reduce survival of free-living birds. For example, disrupted nocturnal activity could lead to poorer migratory decisions, such as departure in suboptimal migratory condition or more inefficient or riskier stopovers. However, further research is necessary to determine the proportion of birds' migration mortality (Sillett and Holmes, 2002) caused by prior exposure to MeHg and diverse stressors.

My experiment demonstrates carry-over effects of unpredictable food stress-only on nocturnal activity with induction of behavioural changes through time. Individuals from

the stress-only group had stronger increases in nocturnal activity than control birds. Previous studies indicate carry-over effects of stress (Koren et al., 2012; Latta et al., 2016), and others documented that food deprivation during behavioural recording either increased (Biebach, 1985; Gwinner et al., 1988) or decreased migratory restlessness (Fusani et al., 2011). Corticosterone elevation on breeding grounds resulted in earlier migration departure in female black-legged kittiwakes, *Rissa tridactyla* (Schultner et al., 2014). Similar to this study, my results suggest a direct carry-over effect of breeding ground stress exposure on migratory behaviour. However, the physiological mechanism behind it remains undetermined since hormone levels were not influenced by unpredictable food stress treatment, and body condition was not correlated with nocturnal activity. Chronic stressors can influence birds' glucocorticoid and mineralocorticoid receptor expression (Cornelius et al., 2018; Dickens et al., 2009; Zimmer and Spencer, 2014), which may affect behaviour without influencing hormone levels. It could also be possible that the effect of food stress on migratory behaviour diminished during the time of recording, soliciting birds to increase their nocturnal activity as compensation for their low activity earlier in the month. More studies are needed to understand what factors (e.g., epigenetic, neurological) could induce a change in behavioural activity while the apparent physiological state of an individual remains unchanged.

Contrary to other treatments, individuals co-exposed to stress and MeHg decreased their nocturnal activity throughout the recording period. Such behavioural change with time does not conform to the definition of a compensatory effect between MeHg and stress, and may instead indicate an *independent* outcome from the combined exposure treatment. It suggests that MeHg and food stress could influence migration through different mechanisms. For example, a bird's increase or decrease of nocturnal activity through time may indicate either a change of state or a change in their activity given their state, with each treatment potentially influencing their state or activity differently. While body condition and T4 were not associated with nocturnal activity, it could be possible that corticosterone concentration changed with time differently depending on treatment. Interestingly, zebra-finches exposed to both MeHg and unpredictable food stress failed to mount an appropriate corticosterone response under handling stress (McLaughlin 2021). In migratory birds a similar effect could potentially influence nocturnal activity.

Alternatively, the migratory restlessness decrease over time in coexposed birds may suggest an earlier peak in activity for this treatment group, followed by a decline (see Seewagen et al., 2019). This possibility is supported by the higher fecal corticosterone metabolites in coexposed birds, which were previously associated with earlier departure from a stopover site (Eikenaar et al., 2017). Overall, further studies would be required to elucidate how MeHg and stress interact within an organism.

During the month of recording, MeHg exposure had a higher parameter estimate compared to other effects. This was not statistically significant ($p = 0.057$) possibly due to the low sample size and high behavioural variability between night and individuals. However, I still interpret it as an important overall finding, biologically relevant to the birds. The significant 3-way interaction may not only suggest that MeHg exposure influenced the stress × time interaction, but it can also obscure a MeHg main effect. An increase in nocturnal activity duration in MeHg exposed birds was also observed on the nights prior to blood and fecal collection. Thus, MeHg exposure may affect some specific nights only and be less influential throughout the full recording duration. Such effects support reports of MeHg exposure causing earlier departure of yellow-rumped warblers from a release site (Seewagen et al., 2019) and zebra finch hyperactive behaviour (Swaddle et al., 2017). Conceivably, MeHg may not directly affect migratory behaviour but instead influence it through indirect action on corticosterone levels. This may explain partially why I was only detecting a trend of a main effect of MeHg exposure in this experiment. More studies are needed to significantly link the potential main effect of MeHg on migratory behaviour.

While the model presented here was the best among all others tested, the model had some autocorrelation that could influence the significance of fixed effects. Other model structures without this issue, but containing other issues, lead to similar predictions. Additionally, the model's low marginal R^2 (Table 4.1) indicates a weak effect of treatments on the nocturnal activity overall, potentially caused by the time between the end of exposure and the start of migratory behaviour. In comparison, the model's high conditional R^2 points to nocturnal activity data distribution being strongly influenced by individual- and night-level variability (see also random effects standard deviation). In the

wild, song sparrows are short-distance migrants (mean travelling distance of 850 km; Davis and Arcese, 1999; Kelly et al., 2019) that will migrate during one or two nights before stopping over for one to two nights. In captivity, song sparrow nocturnal migratory restlessness is highly variable (Figure 4.2), going from being active all night long to inactive the next night. Such high individual variability associated with my fairly low sample size of 8 individuals per treatment may have reduced the detection of influential fixed effects such as the mercury treatment main effect. I hence recommend a bigger sample size for future similar studies.

I measured hormone levels and body condition as possible mediators of migratory behaviour. Surprisingly, treatment exposure did not influence T4 plasma concentration. This differs from prior studies showing MeHg exposure to affect this hormone in developing birds (Champoux et al., 2017; Wada et al., 2009) and a potential action pathway of MeHg through binding with deiodinase enzyme that is required for T4 synthesis (Pantaleão et al., 2017). It could suggest that MeHg effects on this hormone may vary between developing and adult life-stages. Contrary to my prediction and prior studies (Pathak and Chandola, 1982; Pérez et al., 2016), T4 concentrations did not change during the migration period in comparison to prior time points. However, it should be noted that these studies were mainly done during spring migration, contrary to mine, and different thyroidal mechanisms have been suggested between spring and fall migration periods (Pathak and Chandola, 1982). At the time of the August blood sample about half of the birds had started their moult (mean date of moult start on 5 August) that should have increased their T4 level (Jenni-Eiermann et al., 2002; Vézina et al., 2009). Hence, the measurement at two life-stages requiring T4 actions could have prevented the detection of other seasonal variations. Similarly, plasma T4 concentrations were not correlated with restlessness activity, and treatments were not influencing the relationship. However, due to the loss of samples, the statistical power to assess T4 was low. Hence, my results need to be taken with caution.

The fact that fecal corticosterone metabolites did not increase from May to September contrasts with earlier studies suggesting a role of this hormone in triggering migration (Eikenaar et al., 2015; Holberton et al., 2008; Nilsson and Sandell, 2009) but not with

another paper (Ramenofsky et al., 2017). A decrease in fecal corticosterone metabolites concentration during August, corresponding to the birds' beginning of moult, was only detected in the control group. Such corticosterone downregulation with moult was observed in prior research (Romero, 2002; Romero et al., 1998a). In chapter 3, I demonstrated that MeHg but not stress treatment increased song sparrow moult duration, which could then delay their migratory departure date (Cristol et al., 2014; Kiat and Izhaki, 2016; Kiat et al., 2019b). However, here I demonstrated that such delayed departure is unexpected given MeHg exposed birds higher nocturnal activity. Hence, birds exposed to MeHg-only or with stress co-exposure may depart with uncompleted moult and-or at suboptimal moment, possibly resulting in decreased in migration success as observed in Ma et al., (2018a).

Migratory activity was positively correlated with fecal corticosterone metabolite levels, similar to other studies (Eikenaar et al., 2014a). This supports the hypothesis that corticosterone is involved with energy fueling during migration (Cornelius et al., 2013; Falsone et al., 2009; Wingfield et al., 1990). However, it is not yet understood if glucocorticoids rise because of the migratory behaviour (*flight support hypothesis*; reviewed in Bauer and Watts, 2021) or if the rise of glucocorticoids induces the behaviour (*departure stimulation hypothesis*; reviewed in Bauer and Watts, 2021). Treatment groups did not affect the positive relationship between fecal corticosterone metabolites and nocturnal activity duration, suggesting that if the *flight support hypothesis* is true, MeHg and stress have direct impacts on migratory behaviour that is then transferred to fecal corticosterone metabolites. Conversely, if the *departure stimulation hypothesis* is true, MeHg and stress effects on migratory behaviour are indirect, through effects on corticosterone levels. It is important to note that the support and departure hypotheses are not mutually exclusive, so MeHg and stress could each have both direct and indirect effects on migratory behaviour. Overall, more research is needed to better understand the pathway through which MeHg influences corticosterone.

4.5. Conclusion

In summary, I determined that unpredictable food stress, either alone or with MeHg exposure, has carry-over effects on nocturnal activity in captivity. These effects on migratory behaviour appear unrelated to T4 hormone level or body condition but could be associated with corticosterone regulation. The differences in nocturnal activity between groups suggest that stressors and toxicants exposure on breeding grounds could have direct and indirect carry-over effects that have the potential to affect the fall migration journey. To fully explain the ongoing decline of migratory songbird populations (Rosenberg et al., 2019b) it is becoming increasingly imperative to identify how stressors and contaminants (McCune et al., 2019) may amplify migration failure and individual mortality. My findings suggest that research and risk assessment programs should quantify and account for long-term carry-over effects of physiological disruption on wildlife behaviour and populations.

4.6. References

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Chapter 5

5. Effects of wintering with methylmercury exposure on spring reproductive onset in song sparrows

5.1. Introduction

Organisms alter their physiology in order to adjust to predictable seasonal or environmental changes. An efficient seasonal transition between annual cycle stages requires both appropriate timing and maintenance of physiological trade-offs in each season. For example, a fast transition from wintering into breeding phenotype would allow earlier migratory departure and arrival on breeding grounds, where early arrival is positively correlated with acquisition of high-quality breeding territories and reproductive success (Hasselquist, 1998; Norris et al., 2004b; Smith and Moore, 2005). On the other hand, arriving too soon in a habitat that cannot yet support the bird or its brood could result in reduced survival or breeding performance (Janiszewski et al., 2013; Ludwig et al., 2006; Visser et al., 2015). Therefore, transitions between seasons or annual cycle stages are under multiple time-sensitive and energetic trade-offs.

Birds' physiological or energetic state strongly influences the onset and duration of annual cycle-stages such as moult and migration (Cristol et al., 2014; Marra et al., 1998; Stutchbury et al., 2011). For example, female greater snow geese (*Chen caerulescens atlantica*) with high pre-migratory condition had an earlier egg laying date than those in low condition, and laying date was negatively correlated with clutch size (Bêty et al., 2003). Also, several hormones are involved in seasonal transitions between annual cycle stages (Bauer and Watts, 2021; Nolan et al., 1992; Pérez et al., 2016; Tonra et al., 2011) and may influence physiological trade-offs between seasons. For example, high testosterone levels hasten transition from winter to breeding but negatively affect fat stores (Ketterson et al., 1991). Thus, any event or compound that perturb either the timing or homeostasis of birds could affect their transition between annual cycle-stages and ultimately influence their performance.

Methylmercury (MeHg) is a ubiquitous neurotoxin (Henny et al., 2002; Hoffman et al., 2009; Scoville et al., 2020) that can negatively affect a bird's energy balance (Ackerman et al., 2019; Adams et al., 2020; Gerson et al., 2019), hormone activity (reviewed in Tan et al., 2009) and reduce its reproductive performance (reviewed in Fuchsman et al., 2017). Birds exposed to MeHg on the breeding grounds have reduced clutch or brood size (Albers et al., 2007; Heinz, 1974; Paris et al., 2018; Zabala et al., 2020). Such outcomes are often assumed to be caused by embryo toxicity (Fimreite, 1974), though MeHg can also affect parents, including changes in reproductive behavior such as decreased rates of courting, mating, and incubating behaviour (Frederick and Jayasena, 2011; Hartman et al., 2019; Thaxton and Parkhurst, 1973). However, it is often not clear if these effects on physiology and behaviour are directly caused by exposure at the breeding site or if exposure at wintering sites could affect reproduction via carry-over effects. MeHg exposure is known to induce carry-over effects on survival (Ma et al., 2018a), hormones and behavior (chapter 4) and on breeding site blood THg concentration (Fort et al., 2014; Lavoie et al., 2014b). In general, though, effects of MeHg on seasonal transitions remain understudied.

MeHg exposure on wintering grounds could impact a bird's breeding onset and performance through its neurotoxic effects and potential carry-over effects on hormones. In spring, the increase of day length stimulates numerous neuroendocrine events for birds to transition between winter phenotype and spring reproductive condition (reviewed in chapter 1, part 1.1.2). Additionally, the spring onset of reproductive condition is associated with several forms of neural plasticity, including increased neuronal recruitment to the song-control brain region HVC (Alvarez-Borda and Nottebohm, 2002) and increases in HVC size (Airey et al., 2000; Nottebohm et al., 1981; Pfaff et al., 2007). These changes are thought to be associated with singing performance that is a key part of reproductive behaviour in songbirds. While no study so far has demonstrated effects of MeHg exposure on HVC, there are documented effects on males' singing quality (Hallinger et al., 2010; McKay and Maher, 2012). For this study, I measured several aspects of the song-control system to assess potential impacts of MeHg on singing behaviour.

In this project, I aimed to assess if MeHg exposure on wintering grounds could affect songbirds' seasonal transition to their breeding phenotype. I hypothesised that MeHg could affect breeding onset either through direct neurotoxic effects, and/or indirectly through physiological disruptions that may carry-over after the exposure period. I used song sparrows (*Melospiza melodia melodia*) exposed during three winter months to environmentally-relevant dietary levels of MeHg that are known to induce reproductive deficit. Birds were then photostimulated and euthanized 21 days after the exposure period to assess their progress in transitioning toward reproduction. I predicted that hormone disruption and increased energetic costs would result in a delayed onset of reproductive condition (GnRH neurosecretory cells, testosterone levels, gonad size, cloacal protuberance, brain's song control system). I also quantified other mediators of seasonal transition (body condition, corticosterone, thyroid hormones) to document more completely the potential MeHg effects during this period.

5.2. Methods

5.2.1. Bird capture and housing

Between 2 September and 24 September 2019, I used mist-netting and song playback to capture 33 song sparrows (5 females, 26 males) near Port Rowan, Ontario (42°37'19.2"N 80°27'44.4"W), the University of Western Ontario campus (43°00'24.5"N 81°17'14.0"W), and the university observatory grounds near Elginfield, Ontario (43°11'40.0"N 81°18'34.2"W). All birds were captured under permission from the Canadian Wildlife Service, Environment and Climate Change Canada (Scientific Permit CA0244). Soon after capture, I transported the birds back to the Advanced Facility for Avian Research (AFAR), located at the University of Western Ontario, where they were housed indoors, at 20-22 °C, in individual cages. All animal procedures were approved by the University of Western Ontario Animal Care Committee (protocol # 2017-161). A few days after capture, three birds unexpectedly died or were euthanized, while a fourth bird (control treatment) died from unknown causes during the course of the experiment in early January.

For the first several weeks following capture, each of the remaining 29 birds were kept under the same conditions of natural autumn declining light (light schedule was updated every week). On the 3 and 18 October, all birds received a sub-cutaneous injection of anti-parasitic treatment (ivermectin, 0.025 μL injected as concentration of 0.2 mg/mL). On 9 December 2019 I changed the light schedule to a constant winter photoperiod of 9 h light and 15 h dark (9L:15D). This light schedule remained consistent throughout the entire treatment exposure and was then switched to a summer photoperiod condition (16L:8D) at the end of the treatment exposure period on 2 March 2020. This summer photoperiod remained consistent throughout the post-exposure period of the experiment. I chose to use immediate photostimulation rather than gradual increase in day-length to give all of the birds a synchronized cue for the development of reproductive functions, as commonly done in studies on avian photoperiodism (Farner and Wilson, 1957; Appenroth et al., 2020).

Under all circumstances, birds were kept with *ad libitum* access to water and food. During the first 8 weeks after their capture, the birds' diet was a mix of seed (Living World Premium Mix for Budgies parakeet seed) and ground Mazuri (small bird diet). This long pre-experimental period allowed birds to habituate to captivity and to complete their natural moult before the experiment started. On 18 November I began gradually changing their diet to a nutritionally complete agar-based synthetic diet, by mixing the seed with the synthetic diet. The agar-based diet composition was similar to that described in prior chapters. This diet became the birds' main food starting on 30 November until the end of experiment. I replaced the food every day with 25 g of freshly prepared synthetic diet and once a week I gave the birds an additional 5 g of uncontaminated blended eggs.

5.2.2. MeHg treatment

Each bird's sex was first identified by polymerase chain reaction (PCR) of sex-specific genetic markers, and later confirmed by gonad extraction post-euthanasia. I pseudo-randomly assigned the birds into one of two experimental groups, balancing for sex: 14 (3 females, 11 males) control birds received uncontaminated agar-based diet, while 15 (2

females, 13 males) birds were exposed to MeHg through their agar-based diet. I started the birds' MeHg exposure on 2 December 2019, with the diet treated with methylmercury chloride (MeHgCl) at 0.22 ± 0.017 $mg.kg^{-1}$ (mean ± SD) wet weight (w.w.), corresponding to 0.68 $mg.kg^{-1}$ dry weight. I chose this dose to be close to environmentally relevant levels measured in insects in contaminated areas (Abeysinghe et al., 2017; Brasso and Cristol, 2008; Ortiz et al., 2015), but at the same time to be within the range of exposure high enough to induce reproductive dysregulation. Indeed, the effective concentration threshold resulting in 20% decrease in reproduction (EC_{20}) was 0.16-0.75 $mg.kg^{-1}$ w.w. in diet of small-medium bird (Fuchsman *et al.*, 2017). A similar dose resulted in several physiological effects, documented in prior chapters. For each batch of food made, a 1g sample was collected for later THg analysis. The birds were maintained under control or MeHg diet treatments for three months (91 days). On 2 March 2020, corresponding to the first day of photostimulation, all birds received only the uncontaminated agar-based diet for the remainder of the study. I monitored the birds for an additional 24 to 26 days post-exposure before they were euthanized. The timeline of experimental treatment and manipulation is illustrated in Figure 5.1.

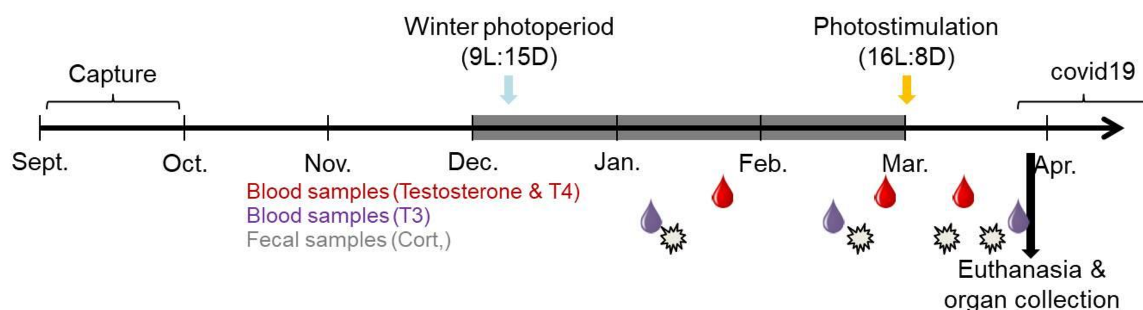

Figure 5.1: Experimental timeline in 2019-2020. The grey rectangle indicates the period of MeHg exposure. The light grey stars indicate dates of fecal samples. The drop symbols indicate dates of blood sample collection for different hormones indicated by colour: red drops for testosterone and T4, and purple drops for T3. I also took an initial blood sample from all birds on 25-28 September to confirm the birds' initial low THg levels. I also measured body mass every 2 weeks, not represented in the figure. The covid19 bracket indicates how the pandemic and university closure forced me to terminate this study sooner than expected.

5.2.3. Blood collection

I took an initial blood sample from all birds on 25-28 September 2019, before the experiment started to confirm the birds' initial low levels of THg. Then starting on 9-10 January 2020, I took blood samples every 2 or 4 weeks (Figure 5.1). To collect blood samples, I punctured the birds' wing vein with a needle and collected 50-200 µL of blood into heparinized microhematocrit tubes. If samples could not be centrifuged quickly, they were kept on ice or in a fridge. Within 5-90 min of collection, samples were centrifuged to separate plasma from red blood cells and I measured hematocrit as the percentage of blood volume made up of red blood cells. Plasma samples were then stored at -80 °C for several months and moved to -30 °C in September 2020 until analyzes.

I used plasma samples for hormone analyzes (androgen, T3 or T4) and red blood cells for THg measurement. Blood samples for androgen hormones (hereafter testosterone) and T4 analyzes were collected on 23-24 January 2020 (day 53-54 exposure), 27-28February 2020 (day 88-89 exposure) and 12-13 March 2020 (day 102-103 post-exposure). Blood samples for T3 analyzes were collected on 9-10 January 2020 (day 39-40 exposure), 13-14 February 2020 (day 74-75 exposure) and 25-27 March 2020 (day 115-117 post-exposure). All blood sampling occurred in the morning between 9:00-11:00am, except on 25-27 March for T3 when, due to covid19 and university lockdown, only one person did the blood sampling at different times during the light hours of the days. One week before this last blood sampling, the birds were subdivided into 4 rooms to allow the bleeder to sample one bird in each room at a time. This created a mean gap of 2 h 26 min (min-max: 33 min to 3 h 19 min) between blood sampling in the same room, to reduce any stress effects of experimenter presence. Each blood sample was taken within 10 min of entering the room.

5.2.4. Mercury analysis

Red-blood cells for THg analysis were collected on 25-28 September 2019 for pre-experiment measures, on 23-24 January 2020, on 27-28 February 2020, just before exposure ended, and on 25-26 March 2020 at the end of the experiment. THg analyzes of red blood cells and agar-based food were performed at the Biotron (an ISO 17025 accredited facility) at the University of Western Ontario, London, Canada using standard accredited methods for ultratrace mercury analyzes. Samples were analyzed using a Direct Mercury Analyzer (DMA-80, Milestone Inc., Shelton, USA) following US EPA Method 7473 (EPA, 1998). About 0.06 g of food and 0.02 g of blood samples were thawed at room temperature and placed in nickel boats for DMA analysis. Laboratory quality control samples included a method blank, sample spiked with an aqueous calibration check standard (CCS) and a duplicate every 10 samples. Quality assurance mean ± SD is reported as wet weight (w.w.) concentration for blood and food. The method detection limit was 0.07 ng and method reporting limit was 0.22 ng. The mean recovery of certified reference material (DORM-4 – fish protein; National Research Council Canada, and CRM PACS-3 – Marine Sediment Reference Material) was at 102 %. The CCS and CCS duplicate were at 101% while the initial and on-going precision and recovery was at 102 %. The relative percent difference between duplicate samples was 3% for both food and blood while the relative difference between spiked duplicates was at 2%.

5.2.5. Testosterone analysis

Plasma testosterone concentrations were assessed via an ELISA kit (Salimetrics Assay #1-2402), following the kit protocol. Samples were diluted in testosterone assay diluent depending on their collection time: winter samples had 20 µL of plasma mixed with 55 µL of diluent while post-photostimulation samples had 15 µL of plasma mixed with 60 µL of diluent. Inter-assay coefficients of variation were determined from a homogenized pooled sample along with high and low control samples analyzed on each plate. The inter-assay coefficient of variation was 4.2%. The intra-assay coefficient of variation was

determined from all samples (n = 93) and was 5.1%. Detected concentrations of all samples were within the standard curve excepted for one individual with very high concentration. This sample was then attributed the concentration of the highest standard.

5.2.6. T4 analysis

Plasma T4 concentrations were assessed via an ELISA kit (Arbor Assays, # K050-H5), following the kit protocol for a 100 µL sample format, where 30 µL of plasma sample was mixed with the same volume of dissociation reagent and with 165 µL of assay buffer. I previously validated this hormone assays for song sparrow plasma by using parallelism and accuracy tests. The inter-assay coefficients of variation were determined from a homogenized pooled sample analyzed on each plate. Most detected samples were close to the standard curve lower detection limit leading to large inter-assay differences in calculated concentration and CV. Also, due to a technical issue, one plate did not produce usable results (high inter-assay CV compared to others). I hence removed it, reducing the sample size. Consequently, I obtained an inter-assay CV of 20.4%. This CV was high, but the error is assumed to be random with respect to treatment, sex and date as samples were randomized across plates. When possible, some samples were tested twice (n = 7), I kept the result of the assay with the lowest variation between duplicates. The intra-assay coefficient of variation determined from all kept samples was 19.4%. Any samples with a concentration below the detection limit were assigned the lowest detection value on the plate (n= 23 out of 78 samples).

5.2.7. T3 analysis

Plasma T3 concentrations were assessed via an ELISA kit (Monobind Inc., #125-300). I first validated this hormone assay for song sparrow plasma by using parallelism and accuracy tests. I followed the kit protocol using 100 µL of plasma sample but, when needed, I added some furnished calibrator 0 to the plasma samples to reach the expected volume, and the dilution factor was later accounted for when calculating plasma

concentration. I analyzed samples in duplicates and all samples' concentrations were within the standard curve. The inter-assay coefficient of variation was determined from a homogenized pooled sample analyzed on each plate. I obtained an inter-assay coefficient of 12.9%. Some samples were tested twice (n = 9), I kept the result of the assay with the lowest variation between duplicates. The intra-assay coefficient of variation determined from all kept samples was 10.3% (n = 76).

5.2.8. Fecal collection and corticosterone metabolite analysis

Fecal collections were chosen instead of plasma as it is a more time-integrated measure (Goymann et al., 2002) and allows regular collection with reduced stress for the birds. I collected overnight fecal droppings (following Eikenaar et al., 2014a) at four time points on 13 January (43 days, exposure), 24 February (85 days, exposure), 9 March (99 days, exposure) and 21 March (111 days, post-exposure). Paper sheets were placed on the bottom of the bird's cage beginning at 3:00 pm and were collected on the next morning at 9:30 am. In each room, placing and removing the paper took approximately 15-30 min, minimizing disturbance. In the morning, each collected paper went into a separate plastic bag before being stored at -30 °C until analyzes.

To extract steroids and steroid metabolites from the fecal samples, I used the same protocol as in chapter 4. The extraction protocol recovery percentage was tested by adding a 25 µL of CORT standard from the ELISA kit into 475 µL of 60% methanol tube before following the same extraction protocol and sample analyze. I obtained a recovery of 96.99 ± 8.036 % (n = 4).

I analyzed the fecal corticosterone metabolites concentration using a corticosterone ELISA method (Arbor assay ELISA #K014-H) following the kit protocol for 100 µL format and mixing 44 µL of the sample with 176 µL of assay buffer. All samples were within the range of the standard curve. Some samples were analyzed more than once, I kept the value of the one with the lowest coefficient of variation. The intra- assay coefficient of variation from all kept samples was of 4.67 % (n = 132). The inter-assay coefficient of variation was 14%, determined from a pooled sample analyzed on each of

the 4 plates. I expressed fecal corticosterone data in pg/mL representing the concentration in corticosterone metabolites in the methanol supernatant that was then used as an estimate for the dry fecal concentration.

5.2.9. Body condition

Before the experiment I measured each bird's tarsus length to the nearest 0.1 mm using dial calipers. Starting on 18 September, I measured each bird's body mass once every two weeks with an electronic balance to the nearest 0.01 g. I calculated body condition as the mass relative to an individual's size as body mass (g) divided by tarsus length (mm) and then standardized as a z-score (with mean = 0 and SD = 1) following prior publication (Ackerman et al., 2019). This resulted in a continuous variable with higher values corresponding to bird with higher mass relative to its size.

5.2.10. Cloacal protuberance

To assess morphological changes associated with breeding, I measured changes in birds' cloacal protuberance size during their regular health checks, on 18 February (about two weeks before photostimulation), 4 March (two days after photostimulation), and 21-22 March (19-20 days after photostimulation). In males, the cloacal protuberance is involved in sperm storage and delivery (Salt, 1954; Tuttle et al., 1996; Wolfson, 1954). In songbirds, swelling of the cloacal protuberance changes seasonally in response to photoperiod and androgen levels, reaching its largest size soon after arrival at the breeding site and staying large during the breeding season (Balthazart et al., 1979; Deviche et al., 2000; Wingfield and Farner, 1978), thus its volume is indicative of reproductive status. The length and width of the cloacal protuberance was measured using dial calipers to the nearest 0.5 mm. The volume was then calculated using the equation for an ellipsoid:

$$\left(\frac{4}{3} \cdot \pi \cdot \left(\frac{width}{2}\right)^2 \cdot \frac{length}{2}\right) \cdot 1000 \qquad \text{Eq. 1}$$

5.2.11. Euthanasia and tissue collection

I euthanized the birds using an overdose of isoflurane inhalation, followed by a transcardial perfusion with 0.1 M phosphate buffered saline (PBS; pH = 7.4) followed by 4% paraformaldehyde. I removed the brains and submersed them in paraformaldehyde for 48 h then transferred them into 30% sucrose in PBS for an additional 48 h for cryoprotection. On the fourth day post-euthanasia, I quickly weighed the brains with an electronic scale to the nearest 0.1 mg, and immediately froze them under crushed dry ice before being stored at -80 °C for long-term storage.

Gonads, syrinx and liver were also dissected out immediately following brain removal and submersed in 4% paraformaldehyde during 7-10 days before being weighed and discarded. Liver has an important role in energy balance and contaminant depuration and is known to be affected by MeHg exposure (Eagles-Smith et al., 2009; Guglielmo, 2018; Seewagen et al., 2022). Syrinx mass increases in response to rising circulating androgens in spring (Luine et al., 1980; Tramontin et al., 2000) and is thus another indicator of breeding state development.

5.2.12. Brain processing and immunochemistry

I used a cryostat at -20 °C to cut brains into 40 µm coronal sections. Sections were collected in alternating series. From the parolfactory lobe (LPO) to the tractus septopallio-mesencephalicus (TSM) split, I collected every section, alternating between sections for doublecortin (DCX) and myelin basic protein (MBP) staining. From the TSM split to the anterior commissure, I collected every second section for GnRH staining. Then from the anterior commissure until the caudal end of the telencaphalon I collected every section again, alternating between sections for DCX and MBP staining.

5.2.12.1. Immunohistochemistry

I followed a three-day free-floating immunohistochemistry protocol for DCX, MBP and GnRH staining. The first day of the protocol consisted of blocking of non-specific antibodies to non-specific binding sites. The brain sections were washed twice using a 0.1M PBS. They were then placed on a shaker for 15 minutes in a 0.5% solution of hydrogen peroxide (#216763, Sigma-Aldrich) diluted in PBS. Following the incubation period, the sections were washed an additional three times in 0.1M PBS before being incubated in the blocking agent. I used 10% normal goat serum (#S-1000-20, Vector Laboratories) for GnRH and MBP stained sections, or 10% normal horse serum (#S-2000-20, Vector Laboratories) for DCX stained sections, the sera being mixed in 0.3% phosphate-buffered saline with triton (PBS/T). The sections were then placed in the fridge (4 °C) to incubate overnight in the serum.

On the second day, sections were incubated in primary antibody: anti-DCX IgG (C-18; #sc-8066, Santa Cruz Biotechnology; concentration 1:501), anti-MBP IgG (MBP [12] oligodendrocyte marker; #ab7349 Abcam; concentration 1:251) and anti-GnRH IgG (GnRH; #20075 ImmunoStar; concentration 1:2001), diluted into 0.3% PBS/T. Trays were then incubated for a 1 h at room temperature with agitation on a shaker before being placed in the fridge to incubate for 24 h.

Finally, addition of the secondary antibody and staining visualization occurred on the third day of the procedure. I first washed the sections three times in 0.1% PBS/T, before incubating sections in secondary antibody for 1 h with agitation on a shaker: biotinylated anti-goat IgG made in horse (#BA-9500, Vector Laboratories) at 1:401 for DCX, biotinylated anti-rat IgG made in goat (#BA-9400, Vector Laboratories) at 1:401 for MBP, and biotinylated anti-rabbit IgG made in goat (# BA-1000, Vector Laboratories) at 1:251 for GnRH. I washed the sections another three times in 0.1% PBS/T, and then incubated them for 1 h in avidin-biotin horseradish peroxidase complex (ABC; Vector Laboratories Elite Kit, #PK-4000). After two washes in 0.1% PBS/T sections were visualized with diaminobenzidine solution (DAB; Vector Laboratories, #VECTSK4103). Sections were then mounted onto microscope slides (Fisherbrand Superfrost Plus, #12-550-15), and then serially dehydrated in ethanol, cleared in xylene substitute solvent

(SafeClear^{TM}, # 23-314629, FisherBrand), and protected with coverslips using Permount (#SP15-500, Fisher).

5.2.12.2. Microscopy

All photomicrographs and measurements were taken by an observer blind to the treatment conditions. I aimed to calculate: (a) the number of GnRH-immunoreactive (GnRH-ir) cells in the preoptic area of the hypothalamus, (b) the volume of song-control region HVC as defined by DCX immunoreactivity (DCX-ir), (c) the volume of the song-control region RA (robust nucleus of arcopallium) as defined by MBP immunoreactivity (MBP-ir), (d) the proportion of DCX-ir per field of view in HVC and (e) the proportion of MBP-ir per field of view of the tracts HVC-RA. GnRH-ir slides were examined using a bright-field light microscopy (Zeiss Axiophot microscope) using a digital camera module (SPOT Idea 5.0 Megapixel camera, Model #28.2) attached to it via an adaptor (#HRD076-CMT 0.76X High Resolution Digital C-Mount Adapter). DCX-ir and MBP-ir slides were examined using a bright-field light microscopy (Leica DM5500B objective lenses) coupled with a camera (Leica DFC 420C) and with the Leica Application Suite software.

5.2.12.3. GnRH producing cells

Each microscope slide was analyzed using a 5X objective lens, and a collaborator (Calista Henry) counted the number of GnRH-ir cell bodies in the preoptic area of the hypothalamus (Figure 5.2a, b) in each bird. Because of technical issues when processing the tissue, some sections were lost or damaged. For those sections (52/840 data points), the number of cells was estimated by averaging the cell counts from the sections on either side or by using the cell count from the other hemisphere when it was intact. Cells were counted in sections from the 12th section rostral to the anterior commissure through to the 2nd section caudal to the anterior commissure.

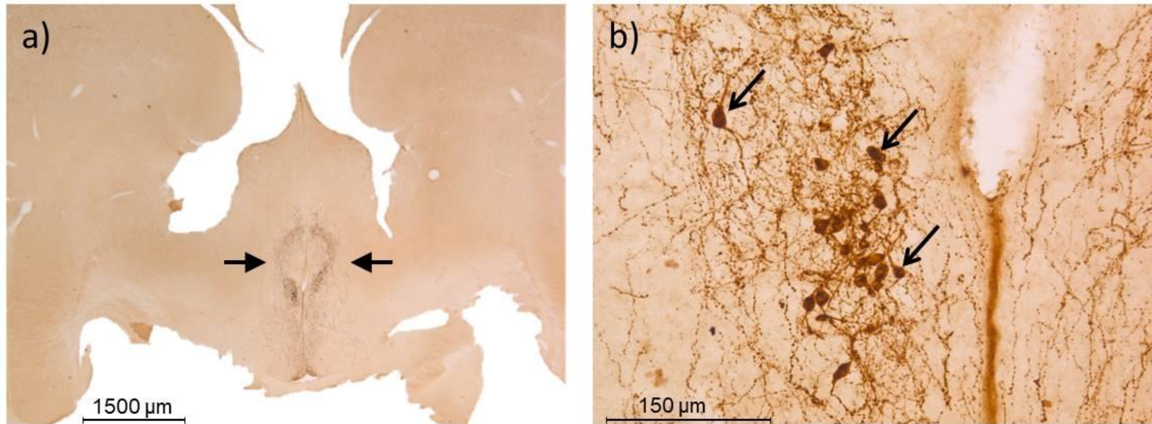

Figure 5.2: Summary of microscope measurements for GnRH-ir cells in the preoptic area of the hypothalamus. a) Example of GnRH-ir section for general position of the areas of interest (within the black arrows). b) Example of GnRH-ir cells (indicated by arrows) that were counted, and immunoreactive fibres.

5.2.12.4. HVC volume and DCX-ir

To quantify the volume of HVC of each brain as defined by DCX-ir, I used the tissue immunostained for DCX with images captured using a 1.25X objective lens (Figure 5.3a). HVC stained for DCX-ir are readily outlined by lower cell density (Hall and MacDougall-Shackleton, 2012; Newman et al., 2010). I quantified the volume of HVC by capturing images from every section that contained the region of interest. Then, using the FIJI version of ImageJ software (Schindelin et al., 2012) I traced the outlines of the area (Figure 5.3b) using the polygon selection tool and a scale of 313.333 pixels/mm. Next, I reconstructed the total volume of HVC using the formula for volume of a frustum (or truncated cone) accounting for sampling intervals of 80 µm. I accounted for any damaged or missing sections by averaging the size of the previous and following sections. Then, I summed the volumes of the frusta to obtain the volume measurement of the entire region. In some cases, HVC could not be identified in immunostained tissue (e.g., in females) or part of HVC region was not collected, precluding an accurate volume measurement. In these cases, the individual was removed from the dataset for this measure. This resulted in a reduced sample size for both the control group (n = 6 males, 2 females) and MeHg

group (n = 6 males, 2 females). For one bird (MeHg treatment), I used the volume of one hemisphere to estimate the second hemisphere.

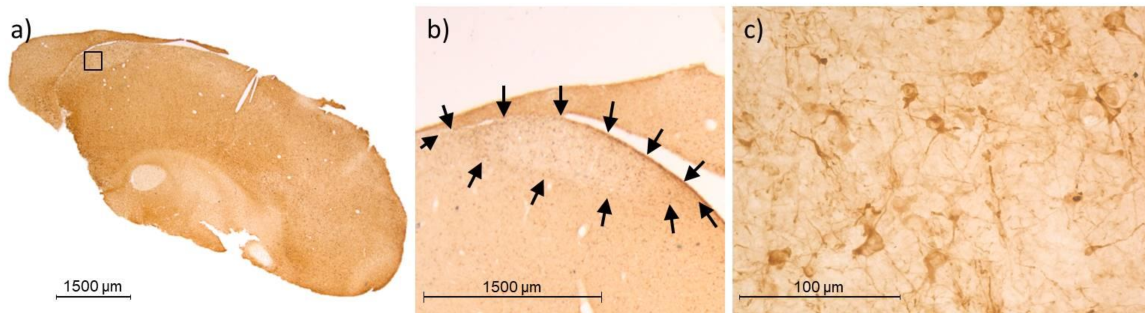

Figure 5.3: Summary of microscope measurements for DCX-ir of HVC. a) Example of DCX-ir section for general position of the areas of interest where pictures have been taken (indicated by the black square). b) Contour of HVC as defined by DCX-ir, indicated by arrows, used to reconstruct the total volume of HVC. c) Higher magnification example of DCX-ir in HVC.

I obtained measurements for the proportion of DCX-ir in sampled images in HVC using photomicrographs captured through the 40X objective lens (Figure 5.3c). I quantified males only since females' HVC were usually too small to obtain a clear view. This resulted in a reduced sample size for both the control group (n =10 males) and MeHg group (n = 10 males). I used the section with the largest cross-sectional area and the 2nd and 4th sections before and after (interval 80 µm between sections for a total sampling area that spanned 360 µm wide in the rostral-caudal axis). I centered the picture taken within the borders of the brain region. If the section was missing or damaged or had very poor staining, I took picture of HVC in the next section, and if that section was also not good, the section's data was not included in the dataset.

Next, I used the ImageJ software to analyze the image. I manually removed any large dust particle (e.g., equal or higher than the size of other cells) from the picture with the polygon tool to prevent it from being counted within the % of immunoreactivity. Similarly, if the field of view contained a large hole, I drew the contour of the area(s) with the polygon selection tool and used the make inverse tool to select the whole field of view except the traced area before extracting the percentage of immunoreactivity within

the selected area. Then, I enhanced the image contrast with 0.2 % saturation and then converted the images into 32-bit gray scale. I used a threshold colour tool using the default setting and, when needed, I manually adjust the threshold to only highlighting DCX stained cells and fibers from background staining. Finally, I used the Area % measure tool to obtain a percentage of immunoreactive highlighted cells per field of view. For one bird (MeHg treatment), I used the immunoreactivity % of one hemisphere as estimate of the second hemisphere.

5.2.12.5. RA volume and MBP-ir

To quantify the volume of RA of each brain as defined by MBP-ir, a collaborator (Yuxie Zhang) used MBP-ir sections, capturing images at 5X objective lens (Figure 5.4a). My collaborator and I followed a similar protocol to that for HVC volume (Figure 5.4b) except that the image scale was 1260 pixel/mm.

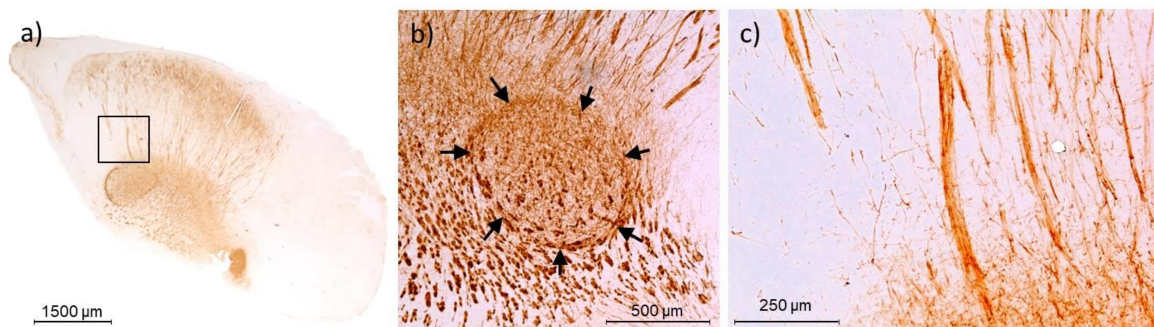

Figure 5.4: Summary of microscope measurements for MBP-ir of RA. a) Example of MBP-ir section for general position of the areas of interest. The black square indicates the position of the picture taken at 10X to quantify the HVC-RA tract. b) Contour of RA as defined by MBP-ir, indicated by arrows, used to reconstruct the total volume of RA. c) Example of MBP-ir in the HVC-RA tract.

I obtained measurements for the proportion of MBP-ir in sampled images of the HVC – RA tract, using photomicrographs captured through the 10X objective lens (Figure 5.4c). I used the sections with the largest cross-sectional area of RA and the 1st and 2nd sections before it, in which the HVC-to-RA tract was most visible (each spaced 80μm for a total

sampling area that spanned 200 µm wide in the rostral-caudal axis). To take the picture, I placed the bottom corner of the field of view against the external contour of the RA area while aiming for the area were the RA-HVC tract was most visible. To reduce the proportion of non-HVC-RA tracts photographed, the field of view did not encompass the approximate vertical middle line of RA line, limiting the potential view to the upper left or upper right corner of RA according to the section orientation (see Figure 5.4c).

Next, I used ImageJ/FIJI software to analyze the image. I manually removed any large dust particles or holes within the tissue with the polygon tool. Then, I did a background subtraction with a rolling ball radius of 25.0 pixels and I converted the images into 32-bit gray scale. I used a threshold colour tool using the default setting to only highlight MBP stained fibers from background staining. Finally, I used the Area % measure tool to obtain a percentage of immunoreactivity highlighted cells per field of view.

5.2.13. Statistical analysis

All statistical analyzes were conducted in R Version 4.0.3 (R Core Team, 2020). All results are presented as arithmetic means ± SE, and level of significance set at $P < 0.05$ because of *a priori* bidirectional predictions. Assumptions of model normality and homogeneity of residual were checked.

5.2.13.1. Hematocrit analysis

To test for seasonal and/or treatment effects on hematocrit I used a linear mixed effect model (lme) via the package *lmerTest* (Kuznetsova et al., 2017). The original full model included the fixed effects of treatment, date of measure as factor and their interaction, and bird ID as random intercept to account for repeated measures. Preliminary analysis indicated no effects of sex, thus the variable was not included in the model. I then removed irrelevant fixed effects following a backward selection on model AIC using the *drop1* function from *stats* package (R Core Team, 2020) before fitting the model with restricted maximum likelihood to extract the results. I determined significant effect with a Tukey post-hoc test using the *glht* function from package *multcomp* (Hothorn et al.,

2008), and I extracted the R^2 with the *r.squaredGLMM* function of the *MuMIn* package (Barton, 2020).

5.2.13.2. Testosterone analysis

To test for seasonal and/or treatment effects on plasma testosterone concentration I first log transformed the data. I then ran a generalised linear mixed model (glmer) via the package *lme4* (Bates et al., 2015), using a gamma distribution without link specification. Simpler models such as lme or models with other family specification led to residual distribution and singularity issues (more details provided in accessible R code). The original full model fixed effects included month of plasma collection, treatment, sex, and the interaction between sex × month, treatment × month. Bird ID was added as random effect to account for repeated measures. Other variables such as tarsus size or cage position were not included since preliminary checks showed no influence. Fixed effects were then removed following a backward selection using the *drop1* function. I then determined significant effects with a Tukey post-hoc test using the *glht* function. Model R^2 value was extracted via *rsq.glmm* function of *rsq* package (Zhang, 2022).

5.2.13.3. T4 analysis

To test for seasonal and/or treatment effects on plasma T4 concentration I log transformed the data and used a lme model. The original full model fixed effect included month of plasma collection, treatment, and their interaction, as well as bird ID as a random effect to account for repeated measures. Other variables such as sex, cage position, and tarsus length were not included following preliminary checks that showed no influence of these data. Fixed effects were then removed following a backward selection using the *drop1* function. Significant effects were determined with a Tukey post-hoc test, and model R^2 was extracted.

5.2.13.4. T3 analysis

To test for seasonal and/or treatment effects on plasma T3, I first tested parametric models (lme, or glmm), with or without data transformation, but none of the models tested met the requirement of residual homogeneity. I hence separated the dataset for each

month and used non-parametric Wilcoxon tests to test for sex or treatment differences within each month. I also tested for difference between months with a Friedman test.

5.2.13.5. Fecal corticosterone metabolite analyzes

To test for seasonal and/or treatment effects on fecal corticosterone metabolites concentration I first log-transformed the data. I then ran a lme model. The original saturated model fixed effects included day of sample collection, sex, treatment, their triple interaction, and cage position as well as the interactions between treatment × cage position and day × cage position. In order to account for the repeated measure bird ID was added as a random effect. I then removed irrelevant fixed effects following a backward selection on model AIC using the *drop1* function before fitting the model with restricted maximum likelihood to extract the results and the R^2 as described previously. Significant effects were analyzed with a Tukey post-hoc test.

5.2.13.6. Body condition

To analyze variation in body condition, I use a lme model. Preliminary analysis indicated that body condition changed non-linearly with time and was better explained by a polynomial function of 6 degrees. The model hence included the fixed effects of treatment, the sextic polynomial transformation of time, their interaction, as well as sex. I added the variable time and bird ID as correlated random intercept and slope to account for the repeated measures. This random structure allows accounting for repeated measures while each individual could have a different slope. This structure was chosen after preliminary checks showing a better AIC value and better model fit when compared with other possible model structure. I then removed irrelevant fixed effects by likelihood ratio test via the *drop1* function. The model results were obtained by fitting the model with restricted maximum likelihood, and each model R^2 was extracted via the *r.squaredGLMM* function of *MuMIn* package.

5.2.13.7. Cloacal protuberance

To test for seasonal and/or treatment effects on cloacal protuberance, I first calculated the % of change in cloacal protuberance from the first measure on 18 February. This step

reduced the noise coming from data variability and measurement error. Then, I ran a *lme* model on the data of males only. I chose to exclude female data from analysis due to the small number of females in the study and their smaller protuberance size. The original model fixed effects included treatment, factorial week of measure, their interaction, as well as body condition and the interaction body condition × factorial week. Bird ID was added as a random effect to account for repeated measures. I then removed irrelevant fixed factors following the same backward selection procedure and results extractions than prior models.

5.2.13.8. Tissue mass and volume (gonads, syrinx, liver, brain)

To test for treatment effects on body tissues I first log-transformed the gonad and liver data to fit the test assumption requirements. For gonads I then ran a t-test on the log-transformed gonad mass of males only to determine the effect of treatment. For the syrinx, I used a linear model (lm) to assess the effect of sex and treatment without interaction. I also used a lm on the log-transformed liver mass as the dependent variable with treatment, sex, and body mass at euthanasia as independent fixed effects. Finally, I used a lm on brain mass to test the effects of treatment and sex without interaction.

5.2.13.9. Brain GnRH

First, I log-transformed the GnRH cell count per bird to conform to test requirements of normality, and then performed a t-test to compare cell count between treatments. In a second step I assessed if other co-variates were related to the number of GnRH-ir cells. I run a linear model (lm) on the log-transformed cell count data and included the fixed effect of treatment, body condition, sex, cage position and the interaction between treatment × body condition, sex × treatment and sex × body condition. After validating the model assumption, I removed irrelevant variable by likelihood ratio test via the *drop1* function.

5.2.13.10. Brain HVC area volume with DCX and DCX-ir %

To assess if MeHg affected HVC volume as defined by DCX-ir, I used the averaged data of each hemisphere for each bird. I first used a Wilcoxon test to assess sex difference in

volume. Then, I selected only males and did a t-test to test for treatment effects. A similar t-test was done on males only to assess treatment difference in DCX immuno-reactivity % per field of view in HVC area.

5.2.13.11. Brain RA volume with MBP and MBP-ir in HVC-RA tracts

To assess if MeHg affected RA volume as defined by MBP-ir, I used the averaged data of each hemisphere for each bird. I first used a non-parametric Wilcoxon test to assess difference between sexes on untransformed data. I then log transformed the RA volume data for males only before doing a t-test to assess differences between treatment groups. I did the same procedure to assess MBP-ir fibers in the HVC-RA tract.

5.2.13.12. Follow-up correlation analysis

In view of the results below, I did a follow-up analysis to try untangling direct and indirect effects of MeHg on some variables. For the last time point of the measures collected, I selected only males and ran a Pearson correlation matrix on the variables DCX-ir % in HVC, log transformed fecal corticosterone metabolites and cloacal protuberance volume, using the *rcorr* function of the *Hmisc* package (Harrell, 2022). I also conducted a Spearman correlation matrix between body condition or GnRH cell count and those three variables.

5.3. Results

5.3.1. THg analysis

On the 25-28 September, all birds had a whole blood THg of 0.0076 ± 0.014 mg.kg^{-1} (mean ± SD; n=30). Throughout the experiment, control birds had a THg concentration in red-blood-cells of 0.051 ± 0.065 mg.kg^{-1} w.w..On the contrary, MeHg exposed birds had a red-blood-cells level of 10.85± 1.24 mg.kg^{-1} at the end of exposure on 27-28 February 2020 and of 5.22 ± 1.93 mg.kg^{-1} at the end of the experiment on 25-27 March 2020. Because in this species whole blood THg level is equivalent to approximately 48.8 % of red-blood cells levels (Bottini et al., unpublished data), MeHg exposed birds would have

a whole blood equivalent THg level of approximately 5.29 mg.kg^{-1} at the end of exposure period.

5.3.2. Hematocrit

Variation in hematocrit was not explained by treatment, or the interaction of treatment × date of measure (all $p>0.1$), hence these fixed effects were removed from the final model. However, hematocrit changed significantly with date, increasing following photostimulation (Table 5.1; Figure 5.5).

Table 5.1: Final model on hematocrit % variation, selected after variable removal. Indication of [date] indicates which factor group of the data is compared to the reference group (e.g., first measure on 9-10 January). Final model's significant variables ($p<0.05$) are displayed in bold and italic.

a. Hematocrit variation				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	49.094	0.53	93.29	<0.001
Date [23-24 Jan]	0.59	0.48	1.22	0.22
Date [13-14 Feb]	***1.54***	***0.48***	***3.24***	***0.0016***
Date [27-28 Feb.]	***1.043***	***0.448***	***2.17***	***0.033***
Date [12-13 Mar.]	***4.55***	***0.48***	***9.56***	***<0.001***
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	5.006	2.24	30	
Residuals σ^2	3.30	1.82		
Intraclass-correlation coef. (ICC)	0.60			
Observations	144			
Marginal R^2 / Conditional R^2	0.24 / 0.70			

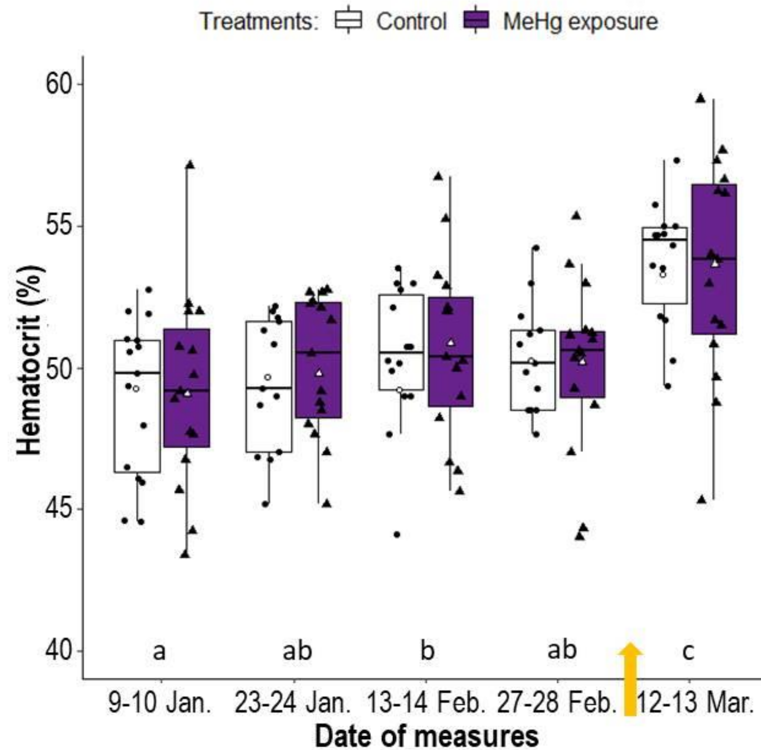

Figure 5.5: Hematocrit (percent of blood volume composed of red blood cells) variation with month and MeHg exposure. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate the individual's treatment, where the control group is indicated by black circles in white boxplots (n = 13-15 in each month), and the MeHg exposed group is indicated by black triangles in purple boxplots (n = 15 in each month). The white symbols indicate the mean hematocrit for each treatment group. The yellow arrow indicates the end of exposure and change in photoperiod. Lower case letters indicate significant differences between dates. Dates that share the same letter are not significantly different from each other.

5.3.3. Testosterone

Testosterone was not affected by treatment (Figure 5.6). The final model included the interaction of month × sex (Table 5.2). While female testosterone levels did not

significantly differ across collection dates (Tukey post-hoc test, p > 0.1), male samples collected on March 12-13 had higher concentration of testosterone than prior time points (Figure 5.6; Tukey post-hoc test, p < 0.01). The two time points during exposure were not different from one another (Tukey post-hoc test, p > 0.1).

Table 5.2: Result of plasma testosterone concentration, glmer final models, selected after variable removal. Indication of [sex] and [month] signals which factor group of the data is compared to the reference group (e.g., females or month of January). Final model's significant variables (p<0.05) are displayed in bold and italic.

a. Plasma testosterone levels variation				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	0.23	0.012	18.710	< 0.001
Month [February]	-0.0019	0.015	-0.13	0.90
Month [March]	-0.016	0.014	-1.089	0.28
Sex [Male]	-0.0097	0.013	-0.74	0.46
Month [February] × sex [Male]	0.013	0.016	0.80	0.43
Month [March] × sex [Male]	***-0.034***	***0.015***	***-2.18***	***0.029***
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	<0.001	0.0093	29	
Residuals σ^2	0.012	0.11		
Intraclass-correlation coef. (ICC)	0.36			
Observations	87			
Marginal R^2 / Conditional R^2	0.53 / 0.60			

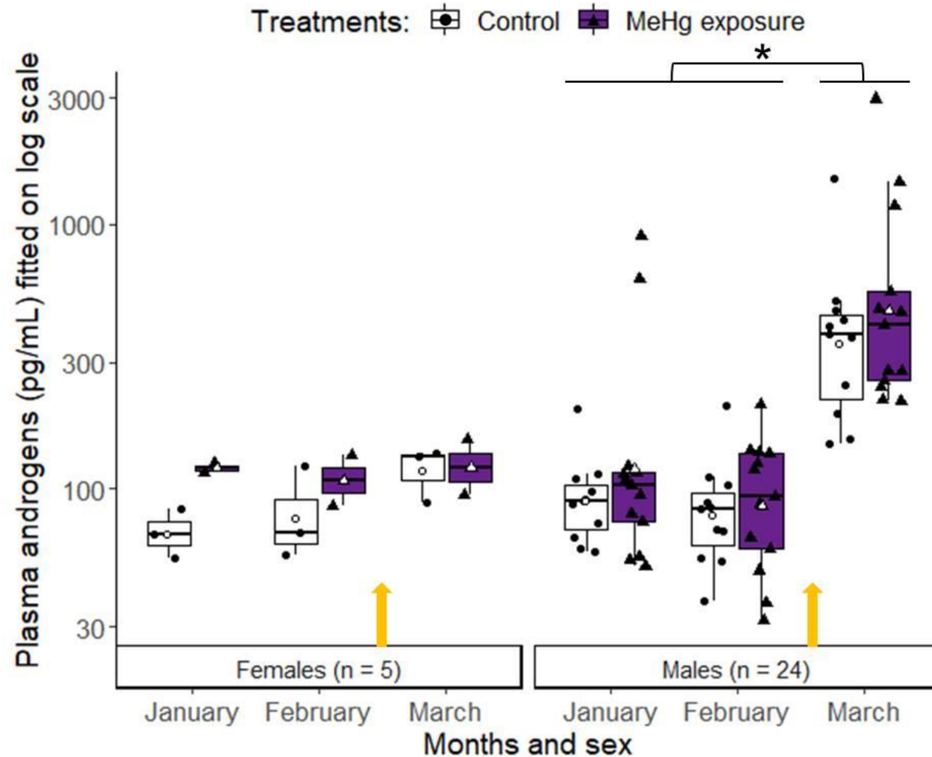

Figure 5.6: Plasma testosterone concentration ($pg.mL^{-1}$) in relation to month and MeHg exposure. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate the individual's treatment, where the control group is indicated by black circles in white boxplots (n = 5 in each month), and the MeHg exposed group is indicated by black triangles in purple boxplots (n = 24 in each month). The white symbols indicate the mean testosterone for each treatment group. The yellow arrows indicate the end of exposure and change in photoperiod. The asterisk indicates that only males show significant increase of testosterone in March (Tukey post-hoc test, $p < 0.01$).

5.3.4. T4 analysis

Plasma T4 was not affected by treatment (Figure 5.7). The final model included only the effect of month (Table 5.3). March T4 plasma concentrations were higher in March compared to the other months (Tukey post-hoc test, $p < 0.001$).

Table 5.3: Result of plasma T4 hormone concentration, lme final models, selected after variable removal. Indication of [month] signals which factor group of the data is compared to the reference group (e.g., compared to January). Final model's significant variables (p<0.05) are displayed in bold and italic.

Plasma T4 hormone levels variation				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	5.5432	0.2776	19.97	<0.001
Month [February]	-0.2259	0.3641	-0.62	0.538
Month [March]	***1.6579***	***0.3560***	***4.66***	***<0.001***
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	0.36	0.60	29	
Residuals σ^2	1.66	1.29		
Intraclass-correlation coef. (ICC)	0.18			
Observations	78			
Marginal R^2 / Conditional R^2	0.265 / 0.396			

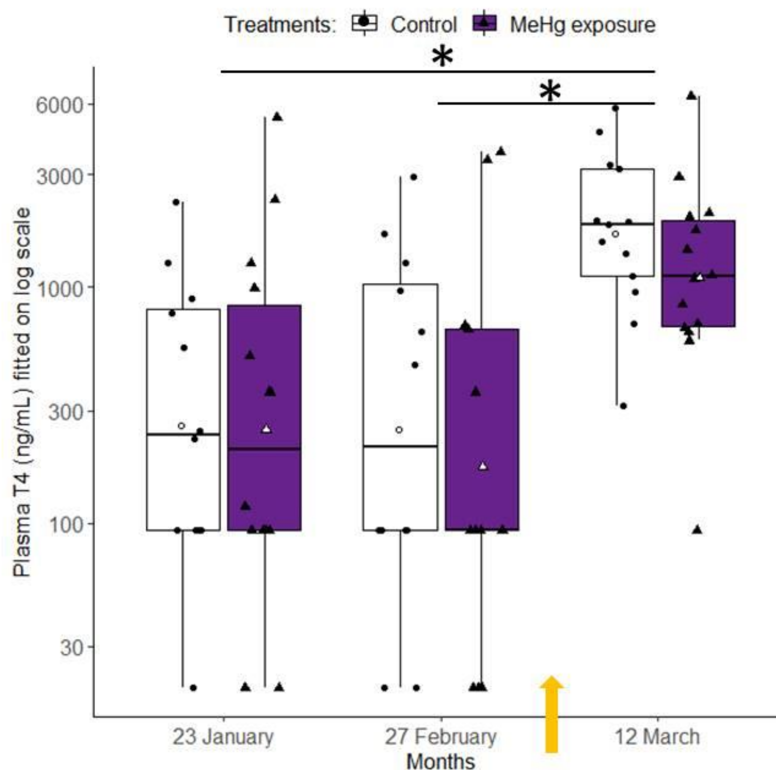

Figure 5.7: Plasma T4 concentration (pg.mL^{-1}) in relation to month and MeHg exposure. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate

the individual's treatment, where the control group is indicated by black circles in white boxplots (n = 12-13 in each month), and the MeHg exposed group is indicated by black triangles in purple boxplots (n = 13-14 in each month). The white symbols indicate the mean T4 for each treatment group. The yellow arrow indicates the change in photoperiod. The asterisks indicate significant difference between months where March T4 concentration increased compared to other months (Tukey post-hoc test: $p < 0.001$).

5.3.5. T3 analysis

Non-parametric analysis of plasma T3 concentration showed no significant difference between months (Friedman test: $X^2 = 1.31$, df =2, $p = 0.52$). Within each month there were no significant effects of sex or treatments (Wilcoxon tests, all $p > 0.1$).

5.3.6. Fecal corticosterone metabolites

The fecal corticosterone metabolites final model included the non-significant main effect of treatment, day of sample collection, the significant effect of cage position and the interaction between treatment × cage position (Table 5.4). The five birds (2-3 per treatment) in the top cages on the cage racks had overall higher fecal corticosterone (log corticosterone: 7.32 ± 0.33 mean ± SD pg/mL, n = 20) than the birds from lower cages (middle = 6.96 ± 0.2883, n = 35; bottom = 7.017± 0.32, n = 40). MeHg exposed birds had a significant corticosterone increase in late March (Tukey post-hoc test, $p < 0.01$) but control birds did not (Figure 5.8).

Table 5.4: Result of fecal corticosterone metabolites concentration, lme final models, selected after variable removal. Indication of [day] or [Cage position] signals which factor group of the data is compared to the reference group (e.g., compared to 13 January, or bottom cages). Final model's significant variables ($p<0.05$) are displayed in bold and italic.

Fecal corticosterone metabolites levels variation				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	6.990	0.093	75.086	<0.001
trt [MeHg]	-0.076	0.108	-0.706	0.482
fday [24 Feb.]	0.038	0.092	0.410	0.683
fday [9 Mar.]	-0.083	0.092	-0.909	0.366
fday [21 Mar.]	0.172	0.094	1.837	0.070
Cage position [middle]	-0.042	0.083	-0.500	0.621
Cage position [top]	***0.239***	***0.097***	***2.470***	***0.021***
trt [MeHg] × fday [24 Feb.]	-0.086	0.127	-0.672	0.503
trt [MeHg] × fday [9 Mar.]	0.072	0.127	0.567	0.573
trt [MeHg] × fday [21 Mar.]	***0.280***	***0.129***	***2.169***	***0.033***
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	0.022	0.15	29	
Residuals σ^2	0.59	0.24		
Intraclass-correlation coef. (ICC)	0.27			
Observations	115			
Marginal R^2 / Conditional R^2	0.318 / 0.505			

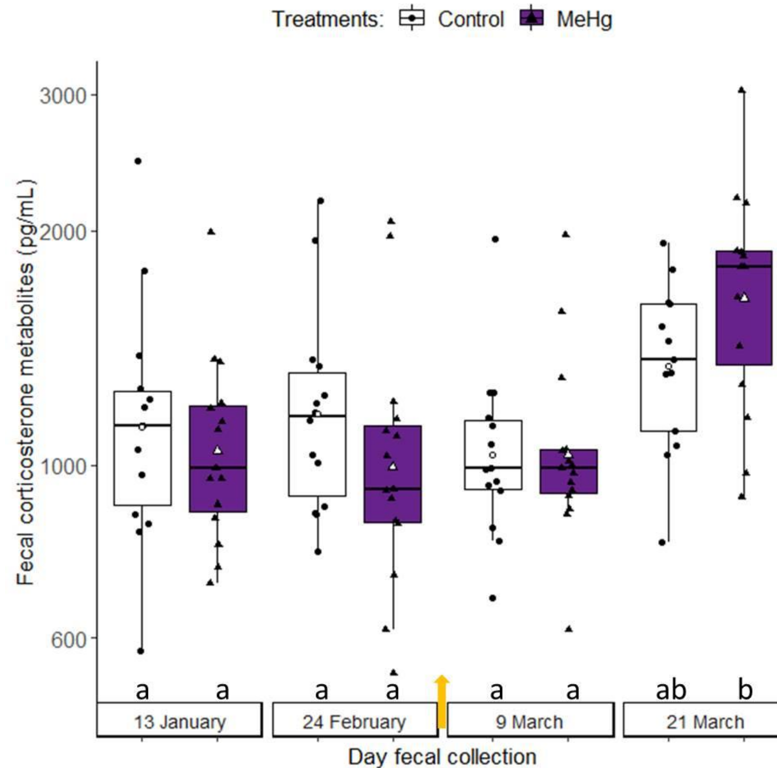

Figure 5.8: Fecal corticosterone metabolites concentration (pg.mL^{-1}) in relation to time and MeHg exposure. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate the individual's treatment, where the control group is indicated by black circles in white boxplots (n = 14-13 in each month), and the MeHg exposed group is indicated by black triangles in purple boxplots (n = 15 in each month). The white symbols indicate the mean corticosterone for each treatment group. The yellow arrow indicates the end of exposure and change in photoperiod. Lower case letters below each boxplot indicate the difference between groups; groups that share the same letter are not significantly different from each other.

5.3.7. Body condition

The final model of body condition included the significant main effect of sex, time, and sex × time interaction (Table 5.5). Neither main effect of treatment nor the interaction

treatment × time significantly influenced the data and were removed from the model. Males had overall higher body condition than females, and each sex varied with time differently (Figure 5.9). A follow-up *lme* model done only on data collected during treatment exposure and post-exposure gave qualitatively similar results (data not shown).

Table 5.5: Result of body condition variation, lme final models, selected after variable removal. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

Body condition variation				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	-0.777	0.229	-3.395	0.002
sex [Males]	***0.933***	***0.251***	***3.722***	***0.001***
week [1st degree]	0.339	2.278	0.149	0.883
week [2nd degree]	***-9.305***	***1.183***	***-7.863***	***<0.001***
week [3rd degree]	***3.720***	***1.181***	***3.149***	***0.002***
week [4th degree]	-1.020	1.183	-0.862	0.389
week [5th degree]	***-2.298***	***1.182***	***-1.944***	***0.053***
week [6th degree]	1.864	1.182	1.577	0.116
sex [Males] × week [1st degree]	4.209	2.499	1.684	0.104
sex [Males] × week [2nd degree]	-1.618	1.299	-1.246	0.214
sex [Males] × week [3rd degree]	***-2.553***	***1.296***	***-1.969***	***0.050***
sex [Males] × week [4th degree]	***-3.022***	***1.298***	***-2.328***	***0.020***
sex [Males] × week [5th degree]	-0.954	1.297	-0.736	0.462
sex [Males] × week [6th degree]	1.081	1.297	0.833	0.405
Random Effects	*Variance*	*Std. Error*	*Correlation*	*N*
Bird.ID τ_{00}	0.20	0.45		30
Week	0.00059	0.024	0.41	
Residuals σ^2	0.24	0.49		
Intraclass-correlation coef. (ICC)	0.46			
Observations	444			
Marginal R^2 / Conditional R^2	0.531 / 0.748			

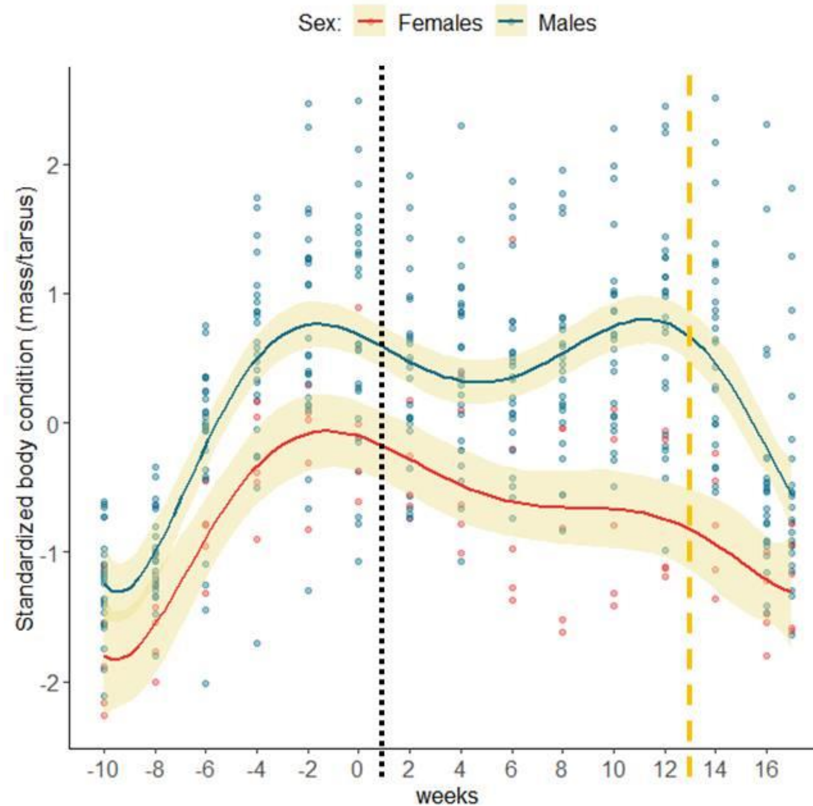

Figure 5.9: Standardized body condition in relation to time and sex. The symbol colours differentiate the females (red) from the males (blue). The black dotted vertical line indicates the start of MeHg exposure on 2 December, while the yellow dashed vertical line indicates the time of photostimulation on 2March. Week #-10 corresponds to measures taken on the 18 September and week # 17 to 27-29 March.

5.3.8. Cloacal protuberance

The final model (using male data only) included the significant effect of treatment and of week of measure (Table 5.6). Other variables such as body condition ($p > 0.1$), and the interaction between treatment ×week ($p = 0.089$) or body condition × week ($p > 0.1$) were not retained in the final model. Cloacal protuberance volume increase with time and MeHg-exposed males had lower change in volume than control males (Figure 5.10).

Table 5.6: Results of final model on males only percentage of change in cloacal protuberance compared to pre-photostimulation measure (18 February). Indication of [day] or [treatment] signals which factor group of the data is compared to the reference group (e.g., compared to 4 March or to control birds). Final model's significant variables ($p<0.05$) are displayed in bold and italic.

a. Percentage of change in cloacal protuberance in males only				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	206.18	20.88	9.88	<0.001
treatment [MeHg]	***-58.64***	***24.75***	***-2.37***	***0.027***
week [21-22 March]	***102.28***	***20.40***	***5.014***	***<0.001***
Random Effects	*Variance*	*Std. Error*	*N*	
Bird.ID τ_{00}	1154	33.97	24	
Residuals σ^2	4993	70.66		
Intraclass-correlation coef. (ICC)	0.19			
Observations	48			
Marginal R^2 / Conditional R^2	0.37 / 0.49			

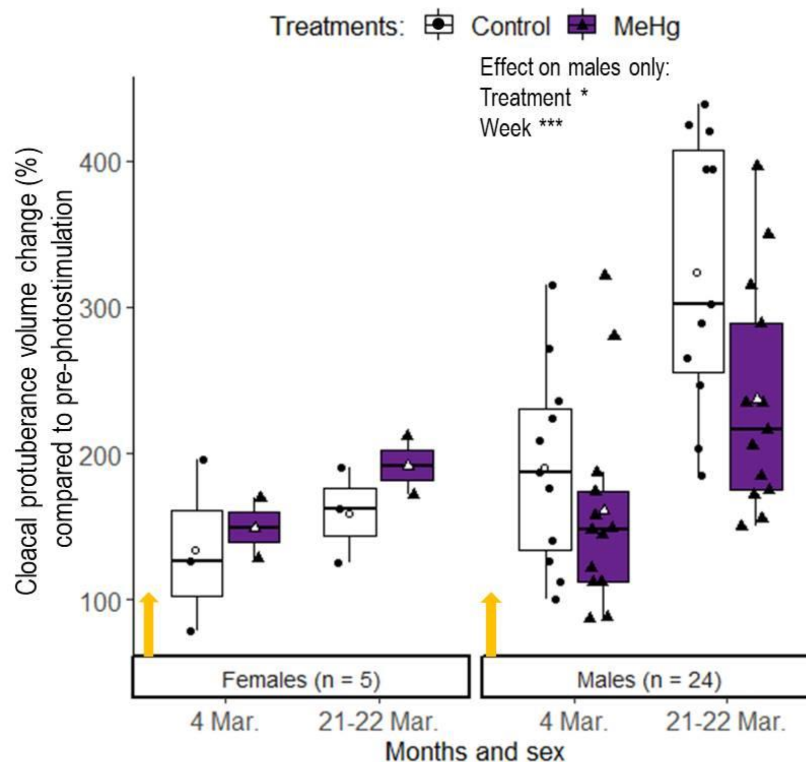

Figure 5.10: Change (%) in cloacal protuberance volume compared to pre-photostimulation measures (18 February), in relation to date and MeHg treatment.

Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate the individual's treatment, where the control group is indicated by black circles in white boxplots (females n = 3, males = 11), and the MeHg exposed group is indicated by black triangles in purple boxplots (females = 2, males n = 13). The white symbols indicate the mean volume change for each treatment group. The yellow arrows indicate the end of exposure and change in photoperiod. The asterisks indicate significant effect of week of measure and MeHg treatment (* $p < 0.05$; *** $p < 0.001$). Note that in this figure, females are only included as visual comparison with males.

5.3.9. Gonads and body tissues

In males, gonad mass was not affected by treatment (t-test: t = -1.18, df = 21.019, p-value = 0.25; Table 5.7).

Syrinx mass was not affected by treatment (lm: estimate = 0.34; standard error = 0.82; t-value = 0.41; p = 0.68), but females had lighter syrinxes than males (lm sex: estimate = 7.15; standard error = 1.082; t-value = 6.60; $p < 0.0001$; Table 5.7), and the model explained a large proportion of data (lm model: $F_{2,26} = 22.43$, residuals = 2.19, adj-R^2 = 0.61; $p < 0.0001$).

The liver mass model indicated no effect of treatment (lm: estimate = 0.094; standard error = 0.063; t-value = 1.492; p = 0.14), but a positive relationship with body mass before euthanasia (lm: estimate = 0.052; standard error = 0.019; t-value = 2.77; p = 0.010). In addition, females had heavier liver mass than males (lm sex: estimate = -0.19; standard error = 0.091; t-value = -2.12; p = 0.044; Table 5.7). The model was significant (lm model: $F_{3,25} = 3.73$, residuals = 0.17, adj-R^2 = 0.23; p = 0.024).

Finally, brain mass was not affected by treatment (lm: estimate = -0.0074; standard error = 0.017; t-value = -0.44; p = 0.66; Table 5.7), or sex (lm: estimate = 0.029; standard error = 0.022; t-value = -1.30; p = 0.21), and the model did not explain variation in the data well (lm model: $F_{2,26} = 0.89$, residuals = 0.045, adj-R^2 = -0.008; p = 0.42).

Table 5.7: Untransformed mass of tissues between treatment and sex. Bold letters and asterisk indicates a significant difference between sexes ($p < 0.05$).

	Tissue mass (mean ± S.D.)			
	Control	MeHg	Males	Females
Brain (g)	0.69 ± 0.048	0.68 ± 0.043	0.67 ± 0.023	0.70 ± 0.047
Liver (g)	0.87 ± 0.17	0.95 ± 0.19	**0.90 ± 0.20**	**0.96 ± 0.085 ***
Syrinx (mg)	16.90 ± 3.38	17.81 ± 3.63	**18.61 ± 2.25**	**11.42 ± 1.53 ***
Testis (mg)	80.25 ± 35.18	98.15 ± 40.66	-	-

5.3.10. Brain GnRH

The first t-test on GnRH-ir cell numbers showed no differences between treatment ($t = 0.40$, $df = 26$, $p = 0.70$; Figure 5.11a). However, when a body condition co-variate was added into a linear model a treatment effect appeared. The final model included the significant main effect of treatment, body condition and the interaction between treatment and body condition (Table 5.8; Figure 5.11b). If females were removed from the dataset, the main effect of treatment and the interaction between treatments and body condition remained significant, but the main effect of body condition became non-significant. Control birds had a positive relationship between GnRH-ir cell number and body condition, while MeHg birds had a negative relationship.

Table 5.8: Results of final model on GnRH cells count in both males and females. Final model's significant variables ($p<0.05$) are displayed in bold and italic.

a. GNRH cells counts in males and females				
Predictors	*Estimates*	*Std. Error*	*Statistic*	*p-value*
(Intercept)	2.145	1.326	1.618	0.119
Treatment [MeHg]	***4.969***	***1.655***	***3.003***	***0.006***
Body condition	***3.154***	***1.320***	***2.389***	***0.025***
Treatment [MeHg] × Body condition	***-4.955***	***1.627***	***-3.045***	***0.006***
Model information				
Residual standard error	0.34			
Observations / df	28 / 24			
Multiple R^2 / Adjusted R^2	0.28 / 0.19			

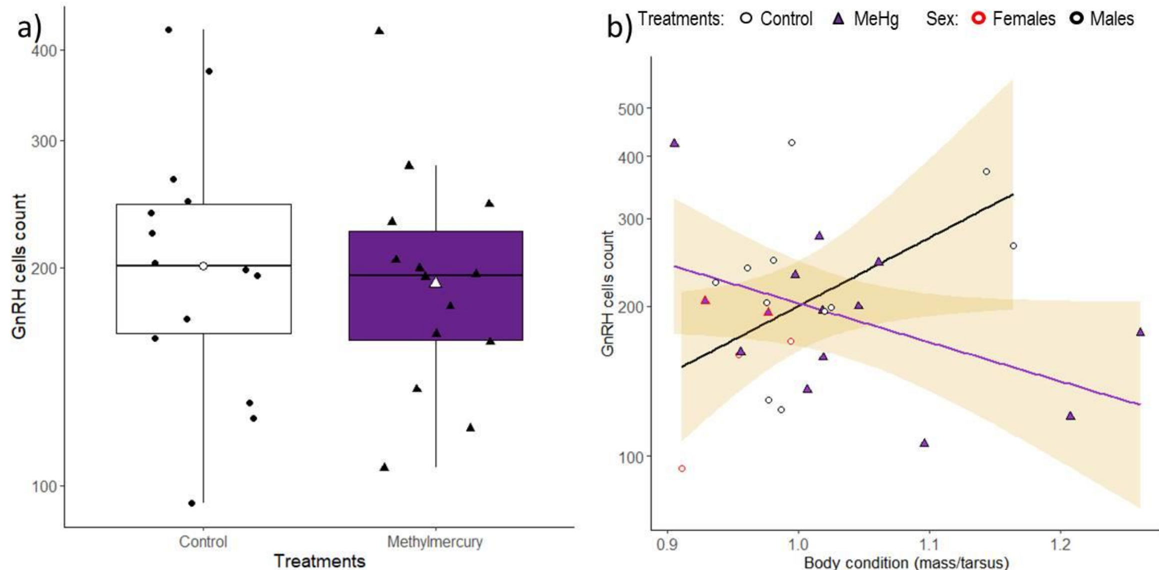

Figure 5.11: GnRH cells count variation. a) Effect of treatments exposure. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate the individual's treatment, where the control group is indicated by black circles in white boxplot (n = 14), and the MeHg exposed group is indicated by black triangles in purple boxplot (n = 14). The white symbols indicate the mean count for each treatment group. b) Effect of body condition. The symbols' outter colour indicates the bird sex, where females are in red and males in black contour.

5.3.11. HVC volume and DCX immunoreactivity

The volume of HVC as defined by DCX-ir was smaller in females (mean ± SD: 101.10 ± 33.77 mm3, n = 4) than males (921.056 ± 155.63, n = 12; Wilcoxon test: W = 0, p = 0.0011). Males showed no difference in HVC volume between treatment groups (t-test: t = -0.89, df = 9.91, p = 0.39). However, there was a significant treatment effect on the percentage of DCX-ir per field of view in HVC (t-test: t = 2.17, df = 15.58, p = 0.04, Cohen's d = 0.97; Figure 5.12). MeHg-exposed males had lower DCX-ir in HVC (12.90 ± 2.99 %) than control males (16.63 ± 4.54 %).

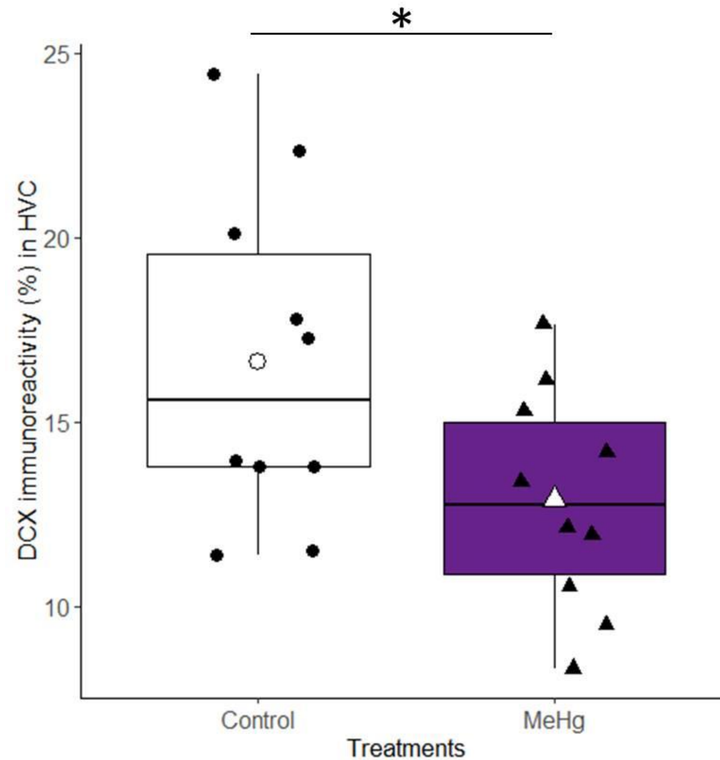

Figure 5.12: Immunoreactivity percentage of doublecortin (DCX), marker of neurogenesis, in HVC area according to treatment exposure. Boxplots indicate 25th, 50th, and 75th percentiles and whiskers indicate range, with individual jittered data points overlaid. The boxplot colour and symbols' shape indicate the individual's treatment, where the control group is indicated by black circles in white boxplot (n = 10), and the MeHg exposed group is indicated by black triangles in purple boxplot (n = 10). The white symbols indicate the mean DCX-ir % for each treatment group. The asterisk indicates significant difference between treatments ($p< 0.01$).

5.3.12. RA volume and MBP immunoreactivity

The volume of RA area as defined by MBP-ir was smaller in females (89.56 ± 47.16, n = 5) than males (346.96 ±72.42, n = 24; Wilcoxon test: $W = 0$, $p < 0.0001$). Volume of RA in males did not differ significantly between treatment groups (t-test: $t = 0.048$, $df = 22$, $p = 0.96$). The percentage of MBP-ir in the tracts between HVC-RA showed no sex

differences (Wilcoxon test: W = 67, p = 0.716) and no treatment difference in males (t-test: t = -1.0055, df = 22, p = 0.33).

5.3.13. Follow-up correlation analysis

The follow-up correlation analysis on males indicated some significant positive associations between body condition and percentage of DCX-ir in HVC (Figure 5.13), but none among other variables (Table 5.9).

Table 5.9: Correlation matrix between variables affected by MeHg treatment: percentage of DCX-ir in HVC area (DCX-ir), gonadotropin releasing hormones (GnRH) cell count, fecal corticosterone metabolites (Cort), volume of cloacal protuberance (CP), body condition (BC). ǂ symbole indicates correlations done with Spearman instead of Pearson test.

	DCX-ir	GnRH	Cort	CP
GnRH	r = 0.19; p = 0.44			
Cort	r = -0.11; p = 0.65	ǂ r = -0.079; p = 0.69		
CP	r = 0.42; p = 0.064	ǂ r = -0.031; p = 0.87	r = -0.18; p = 0.42	
BC	**ǂ r = 0.65; p= 0.0019**	See Table 5.8	ǂ r = 0.24; p = 0.22	ǂ r = 0.36; p = 0.080

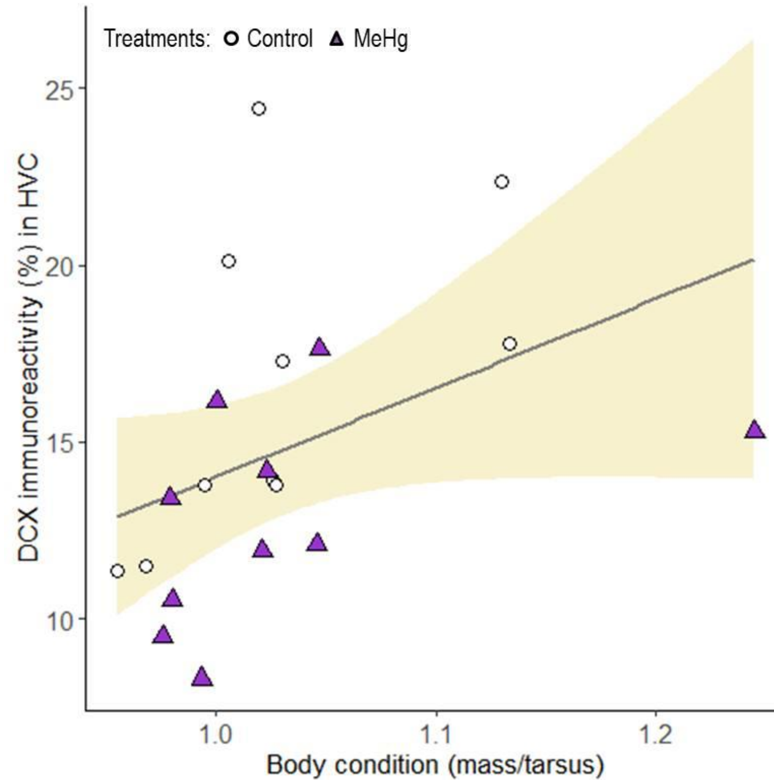

Figure 5.13: Correlation between DCX-ir in HVC area with body condition (mass.tarsus^{-1}; g.mm^{-1}) of males birds, at euthanasia. The symbols' shape and colour indicate treatment group, where controls are indicated in white circles (n = 10), and MeHg exposed birds are indicated in purple triangles (n = 10).

5.4. Discussion

In this study, I aimed to assess if MeHg exposure could affect reproductive onset in song sparrows. Contrary to my predictions, the results indicated no MeHg effect on the timing of spring activation of the HPG axis and most of the other measures. Among the different measures collected regularly, several varied in response to the photostimulation such as hematocrit, testosterone, T4, body condition, cloacal protuberance, and corticosterone for MeHg exposed birds. This indicates that I was able to detect seasonal physiological modifications associated with reproductive onset, but prior MeHg exposure during the winter did not affect most of them. Overall, the results demonstrate that adult male birds

are able to preserve their reproductive physiology in the face of MeHg doses that affect other systems such as moult and migration (chapter 3 and 4). However, the significant effects of MeHg on corticosterone levels, neurogenesis (DCX-ir) in HVC and cloacal protuberance size suggest that MeHg exposure in winter could lead to a potential reduction in mating success even if the timing of reproductive onset was not affected. Indeed, HVC neuronal recruitment is associated with singing performance (Alvarez-Borda and Nottebohm, 2002), that can then affect reproduction and mating success (Reid et al., 2005). Similarly, a large cloacal protuberance is associated with increased sperm competition (Møller, 1988) and disrupted corticosterone influences breeding behaviour and performance (Angelier et al., 2009; Goutte et al., 2010; Silverin, 1998). However, more studies are required to validate my findings with an increased post-exposure period and long-term measurements.

In general, there are seven main non-exclusive explanations for the negative effects of MeHg on adult birds' breeding performance outside of embryo-toxicity and negative developmental long-term effects.

1) Neurotoxic effects, where MeHg exposure may increase neuron apoptosis or affect neurotransmitter receptors (reviewed in Novo et al., 2021; Bottini et al., *in preparation*) and result in the disruption of neural function.
2) Dysregulation of endocrine signals, critical for reproduction (reviewed in Tan et al., 2009; Crump and Trudeau, 2009; Zhu et al., 2000). For example, in birds MeHg affects testosterone (Heath and Frederick, 2005; Jayasena et al., 2011), corticosterone and thyroid hormones (Franceschini et al., 2009; Franceschini et al., 2017; Herring et al., 2012; Wada et al., 2009), and in other taxa MeHg reduces GnRH production and gene expression (Gharaei et al., 2010; Moran et al., 2007; Zhang et al., 2016).
3) At the cellular-organ level, MeHg can reduce gonad mass and induce lesions of reproductive organs (Lundholm, 1995; Pollock and Machin, 2008; Spalding et al., 2000b; Thaxton and Parkhurst, 1973) that could reduce gamete production, fertility and behavior in both male and female birds.
4) Adults' change in physiologic or energetic condition can also be affected by MeHg, such as reduced body condition (Ackerman et al., 2019; Adams et al., 2020; Carravieri et al., 2022), glucose levels (Champoux et al., 2017; Hoffman et al., 2009; Miller et al.,

1980), increased metabolic rate (Gerson et al., 2019) or disrupted lipid metabolism (Seewagen et al., 2022) that may push the bird to reduce energetic investment into breeding or prevent them from breeding.

5) MeHg exposure could reduce secondary sexual signals, (e.g. feather quality, beak colour, singing performance, etc.) that decrease the individual's pairing success or quality of mate. A lower mate quality could in turn lead to lower clutch or brood size or increased nesting failure. For example, MeHg exposure affects feather quality such as colour or mass (Evers et al., 2008; Giraudeau et al., 2015; White and Cristol, 2014), bill colouration (Spickler et al., 2020) and singing quality (Hallinger et al., 2010; McKay and Maher, 2012). Singing or plumage characteristics have in turn been linked with pairing (Hegyi et al., 2010; Pryke and Griffith, 2007; Reid et al., 2004) or reproductive success (Bortolotti et al., 2002; Soma and Garamszegi, 2011; Williams et al., 1993).
6) MeHg exposure can modify the reproductive behaviour and ability of adults to raise offspring to independence. For example, MeHg exposed birds had decreased rates of courting, mating, nest initiation and incubating behavior (Chin et al., 2017; Frederick and Jayasena, 2011; Hartman et al., 2019; Thaxton and Parkhurst, 1973). Exposed individuals are more prone to skip their reproductive season (Heath and Frederick, 2005; Tartu et al., 2013; Zabala et al., 2020), or to abandon or neglect their nest (Jackson et al., 2011; Tartu et al., 2015), and had longer delay to re-nest after clutch loss (Varian-Ramos et al., 2014).
7) MeHg could result in a mismatch between the bird's reproductive readiness and the season, resulting in reduced reproductive success or extra energetic costs of breeding attempt (Carey, 2009; Durant et al., 2007; Visser and Gienapp, 2019). This last possibility has not been well explored but some papers documented an advance in nest-building date (Frederick and Jayasena, 2011), or an advance or delay in female laying date (Costa et al., 2014; French et al., 2010; Hallinger and Cristol, 2011a; Smith et al., 2022).

Overall, all of these seven possibilities may arise from direct exposure effects or could be affected by carry-over effects.

I examined most of these possible MeHg main effects in this study. Contrary to prior research, I found no evidence of MeHg disruption of HPG axis (GnRH, testosterone), or thyroid hormone concentrations, no effect on organs (mass of gonad, liver, brain, syrinx) or on energy balance (body condition). However, I observed some brain neurogenesis reduction in HVC, an increase in corticosterone concentration post-exposure, and a reduced development of the cloacal protuberance in MeHg-exposed birds. These effects could result in seasonal mismatch (explanation #5 above). Because fecal corticosterone metabolites were not correlated with the other measures, it suggests that these negative outcomes resulted from direct independent MeHg effects rather than indirect effects through disruption of one of these measures. With time, these effects could potentially affect the HPG axis, and reduce mating or breeding performance.

Consistent with my results, several authors did not find MeHg effects on androgens such as testosterone (Adams et al., 2009; Franceschini et al., 2017; Pollock and Machin, 2008; Tartu et al., 2013). However, others did (Heath and Frederick, 2005; Jayasena et al., 2011), suggesting that MeHg actions on this hormone may be age-, species- or season-dependent. Song sparrows could also be a species with very high resistance to deleterious MeHg effects on HPG axis and, alternatively, potential damage repair or compensation may have occurred during the post-exposure period (21 days). While these possibilities seem unlikely in view of my prior experiment findings (chapter 4), more research is needed on duration of MeHg carry-over effects and species sensitivity to exposure.

Importantly, my prior study in the same species found a similar post-exposure increase in corticosterone in fall for birds exposed to MeHg and food stress during summer (chapter 4). In both studies, weak to no change in corticosterone levels were observed during exposure, suggesting potential long-term carry-over effects. Increased corticosterone in adults could induce them to have reduced brood size (Angelier et al., 2009; D'Alba et al., 2011), or be more prone to abandon their nest (Angelier et al., 2009; Goutte et al., 2010; Silverin, 1998). MeHg-induced increases in corticosterone following exposure could hence partially explain changes in reproductive behaviour and reduced breeding success in exposed birds (Frederick and Jayasena, 2011; Fuchsman et al., 2017; Hartman et al., 2019; Thaxton and Parkhurst, 1973).

Body condition is known to modulate HPA and HPG responses to hormone signalling (Crino et al., 2018; Grace et al., 2019), as well as reproductive readiness with individuals in better body condition being able to reproduce sooner (Bêty et al., 2003; Devries et al., 2008). Hence, it makes sense that birds with higher body condition had more GnRH producing cells and DCX-ir % in HVC. However, I do not have a biological explanation for the lower GnRH cells count in MeHg exposed birds with higher body condition since treatment did not affect body condition. This result could suggest that maintaining body condition under MeHg exposure may induce some unkown physiological disruptions that are reflected in GnRH cell counts or that resistence to MeHg deleterious effects induced a physiological cost resulting in birds to sacrificing their body condition to achieve reproductive readiness. Hence MeHg effects on GnRH and other measures related to reproductive onset may vary with the physiological state of the individuals, but more studies are required to identify the cause behind it. While the main effect of MeHg on GnRH-ir cell number was significant, the small mean difference between treatment groups suggests a statistical bias due to the significant interaction with body condition rather than a direct effect. No study looked at the effects of MeHg cloacal protuberance and GnRH producing cells in adult birds, so more studies are warranted to confirm these findings.

My observed non-significant effects of MeHg on the HPG axis likely result from an absence of MeHg action on reproductive onset instead of an experimentally too low dose of exposure. Indeed, the experimental birds' whole blood THg concentrations were estimated to peak around 5.29 ppm during exposure. In comparison, research estimated an EC20 reproductive threshold range between 2.1-4.2 ppm w.w. in blood of small and medium birds (Fuchsman et al., 2017) and a complete reproductive failure at 4.0 ppm blood-equivalent concentration (Ackerman et al., 2016a). Hence, the experimental birds' blood mercury concentrations were much higher than the reproductive deficiency threshold value. Additionally, in my previous experiment a MeHg exposure of 0.19 ppm in diet during 3 months of summer, slightly lower than the 0.22 ppm of this experiment, affected moult, migratory behaviour and hormones in fall (chapter 3, 4). Thus, I am confident that the exposure dose was high enough to elicit physiological responses. This suggests that songbirds appear capable of buffering the effects of prior winter exposure so

as to not delay reproductive onset in spring. It also suggests that documented MeHg negative effect on reproduction would be mainly be caused by MeHg exposure on breeding grounds, instead of carry-over effects from exposure on wintering grounds.

Because my study had a low number of females I did not include them in several of the statistical analysis, but my finding of smaller HVC volume in females is consistent with prior studies showing large sex differences in HVC size (MacDougall-Shackleton and Ball, 1999). Importantly, visual comparisons between data for males and females suggest that females may respond differently to MeHg exposure than males on short and long-term time-scales. Thus, more studies are needed to elucidate sex difference in MeHg effects on breeding and reproduction.

In conclusion, I found no effect of MeHg exposure during winter period over the reproductive onset of male birds. However, MeHg did directly disrupt corticosterone levels, cloacal protuberance and DCX-ir % in HVC that could affect breeding success on a longer term. Future studies should incorporate more long-term assessments of MeHg effects post-exposure and assess sex difference in exposure sensitivity.

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Chapter 6

6. General Discussion

My doctoral thesis research aimed to investigate if environmental MeHg exposure and/or unpredictable food stress could exacerbate challenges associated with seasonal transitions and to identify potential post-exposure carry-over effects in songbirds. I used captive song sparrows (*Melospiza melodia*) exposed to MeHg and/or unpredictable food stress for 3 months during one summer (experiment 1) and one winter (experiment 2) and also measured physiological effects during post-exposure observations. My objectives were to: 1) characterized the relationship between unpredictable food stress and MeHg exposure on a toxicokinetic (chapter 2) and toxicodynamic (chapter 3 and 4) point of view. 2) Determine if stress and/or MeHg exposure affects the annual cycle timing of birds and identify the physiological mechanisms (chapters 3, 4 and 5). 3) Assess if carry-over effects occur and what physiological mechanisms may be most affected by them (chapters 3, 4 and 5). Results for each of these objectives are discussed below.

Overall, my main results demonstrated that feathers can be used to effectively monitor environmental mercury since feather mercury concentration strongly reflects blood mercury content at the time of feather growth, but knowledge of species-specific moult phenology is required to properly interpret the feather THg concentrations. Furthermore, MeHg and unpredictable food stress do not often have apparent combined effect and instead appear to have independent effects on birds' physiology and seasonal transitions. However, additive and compensatory effects are observed for fecal corticosterone metabolites and nocturnal activity respectively. Effects on such measures as well as independent effects on other aspects of songbirds' physiology confirmed that MeHg and food stress can have long-term influences on individual's seasonal transitions as well as carry-over effects across seasons. My findings highlight the importance of studying not only co-occurring stressors and contaminants but also evaluating post-exposure effects when assessing potential hazards for wildlife.

6.1. Interaction between food stress and MeHg exposure

6.1.1. Toxicokinetic interaction

In chapter 2, I quantified blood and feather THg loading and depuration throughout the duration of my first experiment. Because I observed a strong positive correlation between feather and blood THg concentration at time of feather moult, I validated the use of feathers in field studies to estimate THg prior exposure, under the condition that species' moult sequence is known. Because blood THg decreased rapidly soon after moult started, this chapter also confirmed that feather mercury sequestration is a major pathway of mercury clearance from the body. However, the blood clearance rate during moult was reduced if birds were still exposed to MeHg when moult started. To my knowledge, no prior research measured blood depuration rate between different exposure situations while accounting for moult start as I did.

Before my thesis project, it was unknown if stressors may influence contaminants MeHg intake, MeHg compartmentalisation or depuration performance. My results reveal no effects of unpredictable food stress on blood THg loading or clearance, but interestingly, food stress slightly increased feather THg sequestration depending on feather type. However, the effect of unpredictable food stress on feather THg sequestration was too low for post-hoc tests to detect THg differences between treatment groups on the same feather type (chapter 2). This finding suggests that field studies measuring THg in feathers would not be strongly impacted by birds' prior stressful experience.

To my knowledge, stress could increase feather THg sequestration via two potential factors: either stress could reduce feather growth rate (as observed in Bateson et al., 2021; Murphy et al., 1988; Swaddle and Witter, 1997) and allow more opportunity for THg sequestration per mm of feather, or food stress may alter the feather composition, increasing the amount of MeHg binding compounds such as keratin, cysteine and reduced glutathione (Crewther et al., 1965; Xu et al., 2019). This last possibility was not studied to my knowledge, but stressors are known to affect feather composition in melanin-based (Fargallo et al., 2007; Galván and Alonso-Alvarez, 2009) or carotenoid-based colouration compounds (Ferns and Hinsley, 2008; Roulin et al., 2008) as well as feather structure

(D'Alba et al., 2014; DesRochers et al., 2009b; Jovani et al., 2014; Pap et al., 2008). Because I did not observe differences in moult duration due to stress treatments (chapter 4), it suggests that unpredictable food stress may alter feather composition. Also, since I observed an increase in feather mass.length^{-1} ratio from P1 to P9 (chapter 4), feather composition may change along the moult sequence. This may partially explain the significant interaction between stress × primary feather type in THg concentration. More studies would be needed to confirm this possible change in feather composition of MeHg binding compound.

While numerous studies previously evaluated how MeHg sequestered in different tissues according to THg or selenium compound (El Hanafi et al., 2022; Golzadeh et al., 2020; Perkins et al., 2017), future studies should investigate if stress treatment may affect MeHg sequestration in non-feather tissues and how this could influence the MeHg:THg ratio over time such as during depuration. Since blood THg sequestration and depuration occurs over time, it would be interesting to see if the MeHg:THg ratio changes within the post-exposure periods in different tissues such as blood and feathers. Since feathers are grown at different times of blood THg depuration (chapter 2), the fraction of MeHg they contain may potentially change with feather type. This would explain some MeHg % differences observed in studies (Thompson and Furness, 1989b; Xu et al., 2019). Such questions would be important to investigate if we want to understand what factors affect the measured THg or MeHg concentration in tissues outside of exposure doses. It would also help validating the use of different tissues (whole blood, red-blood cells and feather types) for exposure estimation in field studies.

6.1.2. Toxicodynamic interaction on birds' physiology

In chapters 3 and 4, I hypothesized that MeHg and food stress exposure may interact in birds through their shared effects on oxidative stress, cell damage, and endocrine dysregulation. Therefore, I predicted that birds exposed to combined MeHg and food stress would have stronger physiological impairments, than the simple effects of stressors and MeHg separately. I measured several physiological components to assess if combined

exposure to MeHg and unpredictable food stress would have synergistic, additive, or compensatory effects. I summarized the main findings of these two chapters in Table 6.1.

Table 6.1: Summary of physiological effects caused by unpredictable food stress and dietary MeHg exposure, obtained from experiment 1. Non-significant but important effects are mentioned by the "n.s." acronym.

	stress	MeHg	MeHg + stress	Period of effect
Body condition	↗	-	No apparent effect	Post-exposure
BMR	↘	-	No apparent effect	Exposure
MMR	-	-	-	-
Fractional metabolic scope	↗	-	No apparent effect	Exposure
T4	-	-	-	-
Cort	↗ with days	-	**varied with days** (No apparent effect then additive)	Post-exposure
Feather mass.length^{-1} ratio	-	vary with feather	No apparent effect	Post-exposure
Wing asymmetry	-	-	-	-
Moult duration	-	↗	No apparent effect	Post-exposure
Migratory restlessness	↗ with days	overall n.s. ↗ but **↗ on some days**	**↘ with days** (compensatory or independent)	Post-exposure

Overall, contrary to my prediction and prior literature (see chapter 1, part 1.6.2 and Table 1.2), MeHg and food stress rarely resulted in interactive effects and instead appeared to affect birds through different physiological disruptions. Indeed, stress mainly affected bird's energetic balance (body condition, basal metabolic rate, metabolic scope, corticosterone) while mercury influenced measures associated with energetic performance (feather quality, moult, migratory restlessness, although not MMR). Overall, my results suggest that wild birds are not at risk of increased deleterious effects when co-exposed to MeHg and unpredictable food stress compared to each independent treatment effect. Some prior studies found either compensatory effects or no apparent effects of treatment exposures depending on the measure (de Jong et al., 2017; Hoffman et al., 2009; Smith et al., 2022; Tartu et al., 2016; Thaxton et al., 1975; Thaxton et al., 1982). Differences between studies could be caused by a difference in the type of stressor, MeHg dose, exposure duration, species or age. Hence, more studies are required to understand what

factors may exacerbate physiological disruption under combined exposure to contaminant and stressors.

In my studies, the independent effects of MeHg and food stress along with the lack of apparent combined effects on multiple measures (Table 6.1) suggest that either the treatments do not share the same mode of action, or that their effects were too weak to induce physiological disruption of shared components compared to control treatment. Indeed, in my study, stress treatment groups had fecal corticosterone metabolite levels similar to the control group during exposure. Thus even if unpredictable food stress elicited some physiological response, it may not be considered a ‘strong’ stressor. Furthermore, it has been suggested that chemical mixture interactions (including antagonism, potentiation, and synergies) usually occur at medium or high dose levels. At low exposure levels they are either unlikely to occur or toxicologically insignificant (SCHER et al., 2012). Indoor studies like mine with manageable stress exposure are more likely to result in compensatory or no-apparent effects. In support of this idea, the studies that found additive or synergistic effects of MeHg and stress on some measures like mortality and reproduction (Fort et al., 2015; Hallinger and Cristol, 2011b; Hill et al., 2008; Hoffman et al., 2009; Roberts et al., 2014; Zabala et al., 2023) assessed stressors that can be more challenging to the birds than unpredictable food stress (chronic hormone injections, high temperatures and storms; chronic low food availability) and could create mortality risk on their own. In these cases, MeHg exposure exacerbated this risk. Also most of these studies occurred during life-stages with high vulnerability to environmental factors (e.g., nestlings and migration). Alternatively, the lack of statistically significant effect between one of my study treatments compared to control group in some physiological measures may have prevented the detection of compensatory or additive effects on the combined treatments. Overall, the term ‘stressor’ is very broad and includes different types of stress that may not act through the same mode of action and may not interact with MeHg similarly as unpredictable food stress. So, caution should be exerted when extrapolating the results of my study to other kinds of stressors.

The only exceptions to these independent or no-apparent combined effects were observed for fecal corticosterone metabolites and nocturnal activity measures. These exceptions

probably occurred because both MeHg and food stress exposure could affect the measures directly or indirectly. While MeHg exposure did not have any main effect on corticosterone, I propose a potential mode of action through which MeHg could directly affect corticosterone concentration but induce effects only during birds' stress response or in a challenging situation such as during migration. In non-avian species, MeHg can bind to glucocorticoid receptors (GR and MR), partially inhibiting the receptors binding capacity and the GR transactivation of gene expression when stimulated with an agonist (Brkljačić et al., 2004; Galigniana and Piwien-Pilipuk, 1999; Spulber et al., 2018). Hence, theses receptors may not fully respond to glucocorticoid signals during challenging situations, potentially inducing a greater secretion of corticosterone as a compensatory mechanism under chronic receptor deficiency. This mechanism also suggests that corticosterone levels would not be affected by MeHg as long as the stress response and/or GR receptors are not activated, partially explaining the lack of MeHg effect during treatment exposure in my study. Hence, stress and MeHg could have direct effects on glucocorticoid secretion or receptor activity respectively. This shared direct effect of treatments on corticosterone during migration may explain why fecal corticosterone metabolite was the only measure demonstrating additive effects.

On the contrary, migratory behaviour is the outcome of multiple factors (e.g., neuronal, endocrine and energetic balance; Lupi et al., 2019), where treatments may either have indirect effects (e.g., via glucocorticoids) or independent effects on this behaviour. This indirect effect in addition to potential other non-measured independent effects (e.g., neurotoxicity) could partially explain the observed compensatory outcome or shift in independent effects with time in the co-exposure treatment group instead of the additive effect I was expecting. Alternatively, the two-month post-exposure duration may have reduced the respective effects of each treatment preventing potential observation of additive or synergistic effect. More research is needed to better predict what would be the outcome of the interaction between two factors.

6.2. MeHg and stress effects on annual cycle timing

My second hypothesis was that MeHg and stress exposure can impact the energy balance trade-off mechanisms of birds as well as their seasonal transitions. MeHg and stress could independently induce oxidative stress, prompt changes in hormone activity and also create damage that require repair, overall taking away energy and nutrients that could be used by birds on traits other than self-maintenance (reviewed in chapter 1). Thus, I predicted disruptions on numerous physiological traits associated with energy balance (body condition, metabolic rates, hormone activity) and a delay in seasonal transition (moult, migratory restlessness and reproductive onset).

The treatment effects on the birds' seasonal transition and physiological performance are summarized in Figure 6.1. Contrary to my prediction I did not observe a delay in the start of season transition with no treatment effects on moult start date and spring reproductive onset (chapter 5). However, my experiments still demonstrated the strong potential for MeHg to impact annual cycle timing in fall, with longer moult duration (chapter 3) and longer nocturnal activity during migratory period (chapter 4). The contrasting results between seasons highlight the importance of reproductive onset and of the hypothalamus-pituitary-gonadal axis in birds that were conserved under exposure level that disrupted other physiological components. While in my second experiment I could not quantify the long-term post-exposure effects, I observed some effects such as increased corticosterone, reduced cloacal protuberance and neurogenesis in the song control system. This could indirectly affect the birds' HPG axis, prolong the bird's transition into its breeding phenotype and/or reduce its reproductive performance later on. To my knowledge, my thesis is one of the few studies suggesting potential consequence of physiological disruption caused by MeHg exposure across seasons. While I focused on individual effect levels, future studies should evaluate what are the outcomes of such long-term effects on populations.

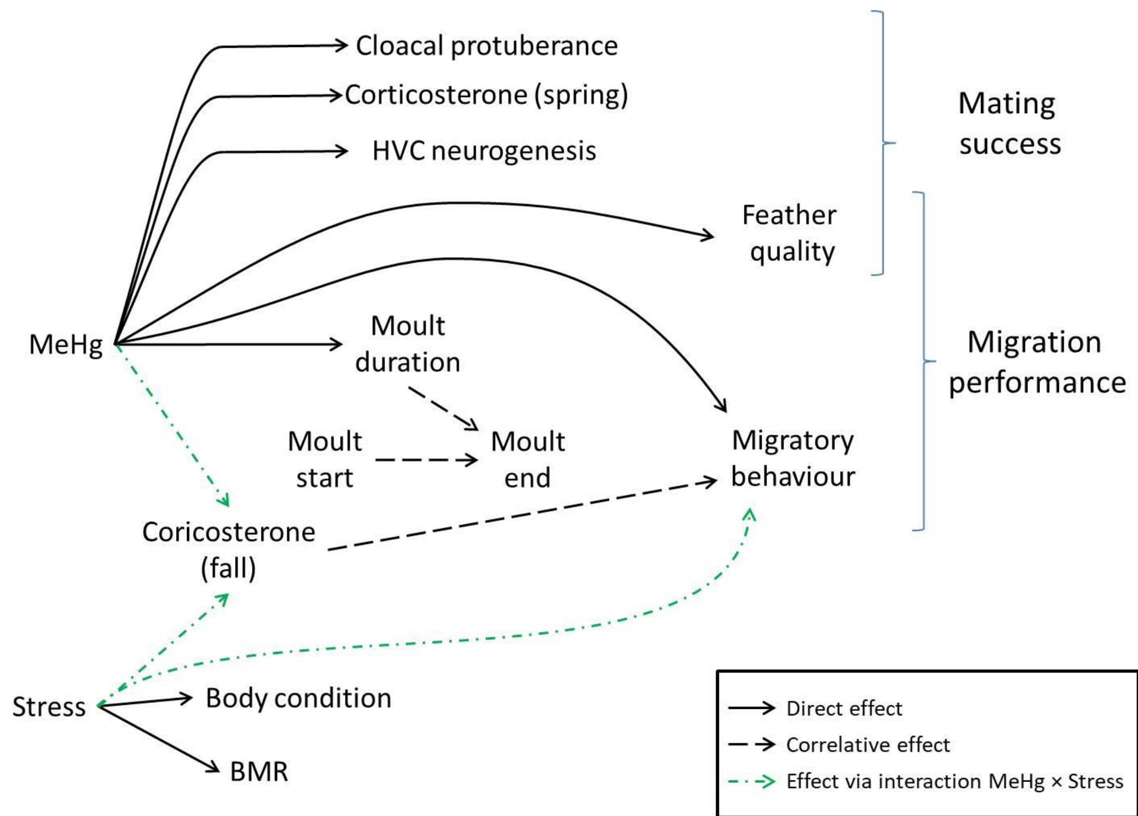

Figure 6.1: Summary of MeHg and stress effects on physiological components that could result in disruption of seasonal transitions and reduced performance in birds.

Contrary to my initial expectation, I was not able to identify clear physiological mechanisms behind MeHg disruption of seasonal timing, although corticosterone disruption appeared to be a major component that would be worth further investigation. To my knowledge, only 4 studies quantified time integrated (fecal and feathers measures) concentration on this hormone and found no or inconsistent MeHg effects (Adams et al., 2009; Herring et al., 2009; Herring et al., 2012; Meillère et al., 2016). Similarly, most studies found no MeHg effects on plasma basal corticosterone levels (e.g., Bowerman et al., 2002; Franceschini et al., 2017; Tartu et al., 2015a) while others found contradicting results (e.g., Maddux et al., 2015; Pollock and Machin, 2009). Thus, my results appear consistent with prior finding since MeHg did not affect fecal corticosterone metabolites during exposure. On the other hand, studies on acute stress-induced corticosterone concentration in plasma either found no MeHg effects (e.g., Tartu et al., 2015b; Thaxton et al., 1982; Wayland et al., 2003) or consistently observed decreasing corticosterone

concentrations with exposure to MeHg (Beck et al., 2014; Franceschini et al., 2017; Moore et al., 2014; Wada et al., 2009) or under combined MeHg and unpredictable food stress exposure (McLaughlin, 2021). These results on stress-induced effects partially support the hypothesis (discussed above) that glucocorticoids receptors activity may be hindered by MeHg binding, although some other factors are apparently influencing this relationship. Thus, more studies about MeHg binding to GR and its consequence on corticosterone secretions under different challenging situations are warranted.

6.3. MeHg carry-over effects across life stages

My last hypothesis and prediction were that treatment exposures could activate energetic trade-off mechanisms of birds. This would result in physiological disruptions continuing even after the end of exposure to stress or MeHg and those disruptions could change with time due to seasonal changes. Among the physiological measures regularly collected over time in chapters 3, 4 and 5, most showed some seasonal pattern (body condition, corticosterone, T4, testosterone, cloacal protuberance, migratory activity, hematocrit) but not all (T3, basal metabolic rate). Interestingly, in both experiments, most of the observed effects of MeHg and food stress occurred during the post-exposure period (Table 6.1, chapter 5). While this is partially due to the study design where moult, migration and reproductive onset were meant to occur within the post-exposure period, post-exposure changes in fecal corticosterone and body mass were not previously assessed in birds. Furthermore, my experiments suggest that MeHg exposure can affect the birds' secondary sexual signals, such as feather quality, neurogenesis in HVC linked to singing performance (Alvarez-Borda and Nottebohm, 2002) and cloacal protuberance that could affect the bird's mating success and reproductive outcome (chapter 4 and 5). More studies are thus required to confirm my observations and to assess the long-term effects of MeHg on birds across seasons.

As noted above, a disruption in physiological balance and seasonal transition could have long-term carry-over effects that might reduce the bird's performance or increase its mortality risk. For example, a delayed moult but longer nocturnal activity during

migration (chapter 3 and 4), in association with changed feather quality may suggest that MeHg exposed individuals could leave their breeding ground in a physiological state or at a time that may not be appropriate for intensive exercising. It could also entail carry-over effects on the follow-up seasonal transition and result in perturbed spring migration date (Fayet et al., 2016). Finally, while unpredictable food stress did not directly impact moult, its influence on body condition and migratory behaviour could also potentially affect the birds' performance throughout the year. Future studies should evaluate what aspect of body condition (lean or fat mass) is the most affected by treatment exposure.

From the different annual cycle stages (summer, moult, migration, wintering and reproductive onset) evaluated in my doctoral research, migration seems to be the period associated with the greatest risk of prior MeHg exposure. Indeed, on its own migration is challenging (Klaassen et al., 2012; Kubelka et al., 2022) and associated with high mortality (Sillett and Holmes, 2002). On top of this challenge, MeHg exposure may increase the energetic challenge during this phase (Gerson et al., 2019; Ma et al., 2018b; Seewagen et al., 2022) in addition to neurotoxic damage (chapter 5). Departing in sub-optimal condition or at a wrong date, as suggested by my data and another study (Seewagen et al., 2019), can reduce migratory performance and survival during the journey (reviewed in Seewagen, 2020). All these factors may explain why birds highly exposed to MeHg are culled in greater proportion during their migratory journey compared to lesser exposed birds (Ma et al., 2018a). This fact may even be more predominant if birds are also exposed to stressor during the prior season. Indeed, as discussed previously, unpredictable food stress is probably a mild stressor compared to other events birds can face in the wild, but I still detected a stress treatment effect 2-3 months post-exposure during migration. Even if I observed compensatory or independent treatment effects on the birds' nocturnal activity, birds exposed to a stronger chronic stressor such as predator presence, immune challenges or poor habitat quality may be more at risk of MeHg deleterious effects during migration than what I quantified.

Additionally, information from both experiments suggests that MeHg effects and associated mortality risk would be greater during spring migration than in fall for North American breeding birds. Indeed, I demonstrated that feather sequestration during moult

is a major pathway for blood THg depuration since whole blood half-life is much shorter in moulting than in wintering birds (chapter 2; Bottini et al. *unpublished results*). Song sparrows do not have pre-nuptial (spring) moult, hence no opportunity to quickly depurate MeHg accumulated during winter. Thus, birds exposed to MeHg on their wintering sites would have to face both the challenge of higher THg blood levels and faster migration pace in spring than fall (Horton et al., 2016; McKinnon et al., 2013; Stutchbury et al., 2009). While I did not quantify migratory restlessness in the second experiment, I observed similar post-exposure increases in corticosterone in fall (1.59 fold increased from mid-May to end September) and spring (1.54 fold increased from mid-January to end of March) for MeHg-only exposed birds. Under the assumption that in this species, corticosterone is positively correlated with migratory in spring similarly to fall, MeHg exposed birds may have similar disruptions in their migratory behaviour in both seasons. However, spring migrating birds may face stronger challenges in spring than fall migration.

Pushing this idea slightly further, my results could help identify species most at risk of MeHg exposure deleterious effects. Indeed, while song sparrows do not winter in South America, other songbirds do. Several South American countries still practice artisanal gold mining that strongly increase MeHg in the environment (Cheng et al., 2022; De Lacerda, 2003; Pirrone et al., 2010; Streets et al., 2017). For example, artisanal and small-scale gold mining represents the largest source of mercury in terrestrial and freshwater ecosystems (Kocman et al., 2017) and most of current atmospheric mercury emission occurs in Asia (49%) and South America (18%; AMAP/UNEP, 2018). Hence, species of songbirds that have weak to no pre-nuptial moult and winter in these countries (e.g., American redstart *Setophaga ruticilla;* chimney swift, *Chaetura pelagica*; Swainson's thrush, *Catharus ustulatus*) could be the most at risk for MeHg deleterious effect during spring migration. Knowing that migratory songbird populations are declining (Møller et al., 2008; Rosenberg et al., 2019b; Stanton et al., 2018), I urge future studies to assess how MeHg exposure may influence the individual's and population's health of those species.

6.4. Future directions, project limitations and concluding remarks

The results from my thesis highlight the fact that physiological and behavioural disruptions induced by stressors or MeHg exposure can last several months after the exposure ended. Hence, more studies are required to better understand the long-term consequences of contaminants exposure on individuals and populations. My research also indicates that MeHg and food stress may not strongly interact, but their effects on corticosterone concentrations and nocturnal migratory activity warrant further studies as well. For example, the assessment of MeHg interactions with stronger stressors than unpredictable food stress is needed.

My thesis experiments also contain several limitations that should be fixed if these studies are replicated. Due to logistical reasons, my second experiment did not include a stress treatment, but some stressors (e.g., poor habitat quality) have been known to affect the spring migratory onset (Marra et al., 1998; McKinnon et al., 2015; Paxton and Moore, 2015) and reproductive success (López-Calderón et al., 2017; Norris et al., 2004b; Stauss et al., 2005). Hence, it would be interesting to replicate this experiment with a stress treatment and a longer period of post-exposure observations. Alternatively, other studies showed that the effects of toxicological challenges are different if they occur concurrently or alternatively (Ashauer et al., 2017), hence assessing the consequence of alternative exposure to MeHg then stressor or the inverse would be interesting.

Another limitation was that my experiments included mostly male subjects. This resulted from the challenges of capturing wild birds. The anecdotal results from the few females in my studies suggest that males and females may respond differently to MeHg exposure, as was suggested in prior studies (Gochfeld, 1997; Robinson et al., 2012). Sex differences in physiological and behavioural responses to stressors or other challenges (Ashley and Demas, 2017; McLaughlin, 2021) have been reported, and females can slightly depurate their blood MeHg into eggs (reviewed in Ackerman et al., 2016b). Hence, males and females are not under the same physiological, behavioural and toxicological constraints. Thus, it would be interesting to confirm some of my findings in female song sparrows.

While my thesis project answers some questions several other aspects remain to be evaluated. For example, my project's results indicated that energy balance and physiological performance could be affected by food stress and MeHg exposure, potentially due to some energetic trade-off and glucocorticoids disruption. As such, it would be interesting to evaluate MeHg effects on immune disruption on the short and long-term. Indeed, MeHg is a known immunotoxicant (reviewed in Fairbrother et al., 2004; Maqbool et al., 2017), and there is a strong interaction between glucocorticoid and immune system (reviewed in Ashley and Demas, 2017; Oppong and Cato, 2015). Avian immunity is known to be affected by energetic trade-offs (Ardia, 2005; Eikenaar et al., 2018; Sanz et al., 2004; Soler et al., 2003) and can decrease with migration (Eikenaar and Hegemann, 2016; Nebel et al., 2012; Owen-Ashley and Wingfield, 2006). Hence, co-exposure to MeHg and stressors or immune challenges (e.g., bacterial infection or parasitic load) may lead to unreported effects on immune balance. Similarly, if migratory behaviour is impacted within two months post-exposure, it would be interesting to do routine behavioural or cognitive tests to assess when behaviour disruption takes place and assess how long it may take before an individual recovers. Finally, I believe that the physiological effects of multiple exposure events over time (e.g., during breeding and during migratory stopovers) would be worth investigating. Indeed, since one exposure could induce long-term carry-over effects, it may possibly sensitize individuals to deleterious effects during a second exposure. To my knowledge, this question has not been studied yet.

In conclusion, MeHg exposure is a contaminant that can have numerous physiological and behavioural effects in the short and long term. While research on this contaminant may be more advanced that for other toxicants, its effects on songbird populations in association with natural habitat challenges are still understudied. With my thesis I demonstrated few additive effects of MeHg and unpredictable food stress and no effect on song sparrow reproductive onset, but the effects on corticosterone, neurogenesis and nocturnal activity may put the individuals at risk during migration. Songbirds are declining all over the world for multiple reasons and environmental pollution, in association or not, with other challenges is certainly a factor enhancing this decline.

Further studies are then required to properly assess where and when those risks take place and adequately inform strategies for wildlife conservation.

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Curriculum Vitae

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	L-SETAC & PN-SETAC Chapters Join Virtual Conference 3rd Place Platform Award PhD, 2021
	The University of Western Ontario, Biology Department, Dr. René R. Roth Memorial Award 2020
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	The University of Western Ontario, Biology Department, Michael Locke Graduate Travel Scholarship, 2019

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