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European Starlings as Sentinels for Health Effects of Urban Air Pollution

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European Starlings as Sentinels for Health Effects of Urban Air Pollution

by

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Abstract

The consequences of exposure to air pollution are widely studied in humans, with urban pollutants associated with a suite of adverse health outcomes. With the complexity of air pollutant mixtures thwarting our full understanding of effects in humans, the consequences to urban wildlife are even less well-understood. The intricate, highly efficient respiratory system of birds makes them more sensitive to airborne toxicants than other vertebrates.

The motivation for this study is to identify sensitive, reliable biomarkers of biological effects of air pollutants using wild European starlings (*Sturnus vulgaris*). This was achieved using two approaches: a field study investigation disclosed the effects of ambient exposure on nestling starlings, whereas experimental exposure of adult starlings to vehicle emissions provided insights under controlled conditions. In both studies, pollutant exposures were measured using several techniques to provide as accurate information as possible. Passive air samplers measured the concentrations of nitrogen dioxide, sulphur dioxide, and volatile organic compounds in ten urban parks in Calgary, Canada during May and June of 2013 to 2015, and during the experiment in May 2016. For the field study, the reproductive success of adult starlings, growth and development of their offspring, biomarkers of oxidative stress, hepatic detoxification effort, and T-cell mediated immune response were evaluated as biomarkers of contaminant effects. The relative contributions of confounding predictors were assessed, while clustering within nest and location were included during analyses. For the experimental study, the same biomarkers were measured in adult, wild-caught, non-breeding starlings, with additional measurements of B-cell immunity, thyroid hormones and histology.

Several responses in nestlings indicated that higher exposures to benzene, toluene, ethylbenzene, xylenes (BTEX) and hexane had physiological costs, which, although subtle, may reduce the resilience of nestlings to cope with additional stressors such as fledging. Similarly, subclinical endocrine and immune changes in experimental birds suggest that higher exposures, or the exposure at sensitive life stages, may have population-level consequences.

Keywords: Air pollution; air contaminants; biomarkers; ecotoxicology; inhalation toxicology; *Sturnus vulgaris*; vehicle emissions

Preface

Chapters 3 and 4 have been published and are included with permission from the copyright holders and co-authors. Chapter 5 has been submitted for publication, and is included with permission from the co-authors. The contributions made by each author are described below.

Chapter 3. This chapter is a modified version of a published manuscript. Reproduced with permission from: North MA, Kinniburgh DW, Smits JEG, European starlings (*Sturnus vulgaris*) as sentinels of urban air pollution: a comprehensive approach from non-invasive to post mortem investigation. *Environ. Sci. Technol.* 2017. Copyright 2017 American Chemical Society. JS designed the original project and supervised MN, who conducted all data collection, analyses and drafted the manuscript. DK provided passive monitors and their analyses as well as insight into the planning and final editing of the manuscript. The full text, published article may be found using the ACS Articles on Request link: http://pubs.acs.org/articlesonrequest/AOR-fTvs67dIxDi6uQ9NQnQc. DOI: 10.1021/acs.est.7b01861

Chapter 4. This chapter is a modified version of a published manuscript, reprinted with permission from: North MA, Rodriguez-Estival J and Smits JEG, Biomarker sensitivity to vehicle exhaust in experimentally exposed European starlings. *Environ. Sci. Technol.* 2017. Copyright 2017 American Chemical Society. MN designed the experimental procedure, drafted the Animal Use Protocol, and executed the experimental study, all under the supervision and guidance of JS and JRE. MN and JS contributed to the drafting and editing of the manuscript. DOI: 10.1021/acs.est.7b03836

Appendix B: This appendix contains Supporting Information from the published Chapter 4, North MA, Kinniburgh DW, Smits JEG, European starlings (*Sturnus vulgaris*) as sentinels of urban air pollution: a comprehensive approach from non-invasive to post mortem investigation. Environ. Sci. Technol. 2017. Copyright 2017 American Chemical Society.

Appendix D. This chapter is a modified version of a manuscript under review by Toxicology Mechanisms and Methods, authored by North MA, Kinniburgh DW and Smits JEG. MN designed and constructed the enclosures and planned the experiment with the guidance and supervision of

JS and DK. All authors provided input during the trouble-shooting and validation of the experiment, as well as the writing and editing process.

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Dedication

For my family,
For supporting my dreams,
even when they change;

Warwick Hastie,
For travelling halfway around the world,
for me;

And to all the people who care enough
to make a difference in this precious world.

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List of Symbols, Abbreviations and Nomenclature

Abbreviation	Definition
[hourly]	Average hourly concentration of a compound
[minute]	Average minute concentration of a compound
$\mu g/m^3$	Unit of concentration (VOCs and BTEX)
AAAQO	Alberta Ambient Air Quality Objectives
AEMERA	Alberta Environmental Monitoring, Evaluation and Reporting Agency
AEP	Alberta Environment and Parks
AER	Alberta Energy Regulator
AQHI	Air Quality Health Index ($PM_{2.5}$ + O_3 + NO_2; Canada, current)
BTEX	Concentration of benzene + toluene + ethylbenzene + xylenes
CASA	Clean Air Strategic Alliance
CFM	Cubic feet per minute, a unit of air movement
CO	Carbon monoxide
CRAZ	Calgary Region Airshed Zone
CYP1A1	Cytochrome P450-1A1 monooxygenase
ECH	Thyroid follicle epithelial cell height
ELISA	Enzyme-linked immunosorbent assay
EROD	7-ethoxyresorufin-O-deethylase (liver CYP1A1 activity)
ESRD	Alberta Environment and Sustainable Resource Development
fT4	Thyroxine concentration (free)
GSSG	Glutathione disulfide
HDJ	Hatch date Julian; days numbered consecutively from January 1
IQUA	Index of the Quality of the Air (Canada, historic)
LSD	Least significant differences
LUR	Land-Use Regression
NAAQS	National Ambient Air Quality Standards (U.S.A.)
NAPS	National Air Pollution Surveillance (Canada)
NDV	Newcastle Disease Virus
NO_2	Nitrogen dioxide

NO_x	Oxides of nitrogen
O_3	Ozone
OECD	Organisation for Economic Cooperation and Development
oxGSH	Glutathione (oxidised)
PCB	Polychlorinated biphenyl
PHA	Phytohaemagglutinin skin test (T-cell mediated immunity)
$PM_{2.5}$ or PM_{10}	Particulate matter (2.5 or 10 µm)
ppb or ppbv	Parts per billion (by volume); unit of concentration for NO_2 and SO_2
rGSH	Glutathione (reduced)
RT	Real-time (for air quality monitoring equipment)
SD	Standard deviation
SEM	Standard error of the mean
SLMI	Scaled Liver Mass Index; size-corrected organ mass
SMI	Scaled Mass Index; size-corrected mass (energy reserves)
SO_2	Sulfur dioxide
SO_x	Oxides of sulfur
SSMI	Scaled Spleen Mass Index; size-corrected organ mass
STMI	Scaled Testes Mass Index; size-corrected organ mass
TBARS	Thiobarbituric acid reactive substances (membrane lipid peroxidation)
tGSH	Glutathione activity (total)
TT3	Triiodothyronine concentration (total)
TT4	Thyroxine concentration (total)
U.S. EPA	United States Environmental Protection Agency
UNECE	United Nations Economic Commission for Europe
VOCs	Volatile Organic Compounds, non-methane hydrocarbons
WHO	World Health Organisation

Epigraph

"You are not exposed to one chemical at a time, but a complex mixture of chemicals that changes day by day, hour by hour, depending on where you are and the environment you are in... In the United States alone it is estimated that over 72,000 different chemicals are used regularly. Two thousand five hundred new chemicals are introduced annually — and of these, only 15 are partially tested for their safety. Not one of the chemicals in use today has been adequately tested for these intergenerational effects that are initiated in the womb."

Theo Colborn

***

"We stand now where two roads diverge. But unlike the roads in Robert Frost's familiar poem, they are not equally fair. The road we have long been traveling is deceptively easy, a smooth superhighway on which we progress with great speed, but at its end lies disaster. The other fork of the road — the one less traveled by — offers our last, our only chance to reach a destination that assures the preservation of the earth."

Rachel Carson, Silent Spring

***

"It always seems impossible until it's done."

Nelson Mandela

Chapter One: **Introduction**

1.1 Problem Definition

1.1.1 Brief history of air pollution

Control of urban air pollution has been on the public agenda since Greek and Roman times, initially recognized as a nuisance needing control to rid areas of the offensive odours and keep affluent areas clean and pollution-free (Phalen and Phalen, 2013). People began to associate air pollution with illness as early as the 13th century, when coal replaced wood as the dominant fuel-source and emissions became increasingly acidic and malodourous (Phalen and Phalen, 2013). The burgeoning European population during the 16–18th centuries burnt ever-increasing amounts of coal for heating and energy, blanketing cities with sulphurous coal-smoke that increased concern for public health and damage to historic buildings. With the Industrial revolution (mid-1800s), the link between exposure to air pollution and human illness was undeniable, supporting legislation to restrict smoke pollution (Chen *et al.*, 2007, Phalen and Phalen, 2013).

Among more recent and memorable global successes, the banning of leaded fuels in vehicles and chlorofluorocarbons as refrigerants stand out as beacons of what is possible. Control of these pollutants was driven by the documentation of serious environmental impacts (e.g., the effects of lead on human and animal health, and the hole in the ozone layer), which motivated the complete change in-, and enforcement of, international environmental legislation.

Maximum permissible levels of specific pollutants have been established based on epidemiological evidence of adverse effects on human health, experimental toxicity studies, and technological advancements that allow the accurate measurement of the concentrations of chemicals in the environment. In their National Ambient Air Quality Standards 'Integrated Science Assessment for lead, oxides of nitrogen, sulphur oxides, particulate matter and ozone and related photochemical oxidants', the United States Environmental Protection Agency (U.S. EPA) discusses how permissible limits are raised or lowered as new evidence comes to light (U.S. EPA, 2008b, U.S. EPA, 2008c, U.S. EPA, 2009, U.S. EPA, 2013a, U.S. EPA, 2013b).

Globally, the concentrations of several air pollutants have been reduced significantly since 1990. These include particulate matter, sulphates, nitrates, ozone, and lead in the U.S. (Costa, 2008) and Europe (Vestreng, 2003, Menz and Seip, 2004). These successes in developed nations are attributable to increased public awareness, international regulatory pressure and environmental treaties like the Gothenburg Protocol prioritizing efficiency and modernization of industrial processes (McLean and Barton, 2008, Kelly *et al.*, 2010). Despite these successes, according to the United Nations Economic Commission for Europe (UNECE) Convention on Long-range Transboundary Air Pollution, many urban areas in Europe, North America and elsewhere are still struggling to maintain air quality within regulatory guidelines for health (Maas and Grennfelt, 2016).

1.1.2 Global watch-bodies / regulators of air contaminants

Globally, there are several agencies responsible for setting (with variable enforcement capacities) recommended threshold concentrations for individual air pollutants; however, many countries simply apply U.S. EPA-published limits (an organization whose authority is being crippled by current political interests). European air quality is legislated by the European Environmental Commission; in Canada both Health Canada and Environment and Climate Change Canada, as well as the respective provincial departments are responsible for the maintenance of clean air, while in the developing world, the World Health Organisation (WHO) has the impossible role of pushing for pollution control.

In 1978, a Canadian committee consisting of federal and provincial representatives developed an index of the quality of the air (IQUA). Five major air pollutants (carbon monoxide, dust and smoke, nitrogen dioxide, ozone and sulphur dioxide) were included when calculating the IQUA, which was categorized based on the effects on soil, water, vegetation, animals, materials, visibility and human health (Myrick and Hunt, 1996). Currently, federal and provincial departments publish an air quality health index (AQHI), that expresses outside air quality (calculated as the weighted sum of particulate matter 2.5, ozone and nitrogen dioxide (Chen and Copes, 2013)) as relative risk to human health (Alberta Environment and Parks, 2017).

The regulatory agencies overseeing air quality in Alberta have been reshuffled several times during the last few years. The pre-2015 government kept environmental monitoring at 'arms-length', divided the responsibility among three, independent agencies: the Alberta Environmental Monitoring, Evaluation and Reporting Agency (AEMERA), Alberta Environment and Sustainable Resource Development (ESRD), and the Alberta Energy Regulator (AER). With the change of government in 2015, environmental monitoring came back under government control, linking environmental well-being and resource development under Alberta Environment and Parks (AEP).

Currently, Alberta air quality monitoring and reporting is overseen by the Clean Air Strategic Alliance (CASA), a multi-stakeholder organisation composed of industry, government and non-governmental representatives. On the local level, they partner with nine airshed zones, including two that incorporate the major urban centres, the Alberta Capital Airshed (Edmonton region) and the Calgary Region Airshed Zone (CRAZ, including Calgary, Airdrie and surrounding areas) (CASA, 2016). These airshed zones are responsible for monitoring and reporting the air quality in their regions, partnering with the provincial government for funding and equipment, and local industry and the public to improve awareness and responsibility (CRAZ, 2017).

1.1.3 Methods of measuring air quality

A complete review of the many methods used for monitoring air quality is beyond the scope of this thesis, however, an overview will be included here, and discussed in more depth in Chapter 2.

There are two fundamental approaches when monitoring air quality: measuring current conditions, and predicting air quality conditions (current, historic or future). Current conditions may be measured using passive samplers that integrate the concentrations of single-pollutants over a period of days to weeks, or, big-data, active monitors that can measure pollutants in real-time, reporting 5-second-, minute- or hourly-concentrations. Handheld 'sniffers' also measure current conditions, although they are designed to detect leaks of toxic gases on industrial sites rather than ambient air quality. Current air quality status is important for public health advisories, and for regulatory purposes.

Air quality predictions use mathematical models to combine known air quality measurements with atmospheric conditions, topography, and other influencing factors to predict pollutants in a specific

location. These can be useful for modelling plumes (i.e., dispersion modelling), to determine areas affected by industrial leaks or for epidemiological studies looking at the effects of air pollution on health. Land-use regression models (LUR) are one such method used in urban areas to facilitate medical research, or to guide future land-use planning decisions. These models require multidisciplinary expertise; knowledge of atmospheric chemistry, long-range transport of pollutants, meteorology, and statistical techniques, among others (El-Harbawi, 2013). There are many models which incorporate factors relevant to specific applications.

Passive samplers have been used in field-applications, and validated against active monitors (Blum *et al.*, 1997, Bytnerowicz *et al.*, 2002a, Bytnerowicz *et al.*, 2002b, Cox, 2003, Roadman *et al.*, 2003, Bari *et al.*, 2015). The time-integrated pollutant concentrations provide valuable data for air quality monitoring, depending on the question to be answered. While passive samplers miss peak levels which might occur with accidental industrial releases, the long-term averages are more relevant for the toxicology of chronic, ambient exposures.

1.1.4 Air contaminants and health in Calgary

The U.S. EPA regulates six, 'criteria' air pollutants (1–6 in Figure 1-1, U.S. EPA (2016b)), the WHO, four (1–4, WHO (2016)). This study focuses on two of these pollutants, namely sulphur- and nitrogen oxides, but also includes volatile organic compounds (VOCs, non-methane hydrocarbons), a group of compounds linked to mobile (traffic) sources that were included in the Convention on Long-range Transboundary Air Pollution in 1991 as being of environmental concern (Anon, 2015, Anon, 2016a). These volatile compounds were also a major driving factor for the Western Beef Productivity study, after high profile public discourse on the ecosystem (livestock, wildlife, human) health impacts of the VOCs released by oil and gas production, transportation, storage, and use by vehicles (WISSA, 2006).

Urban air pollution may include a suite of additional compounds produced during industrial processes, from vehicle exhaust, household items or pest control. These include compounds with demonstrated toxicity to wildlife and people, such as lead from leaded gasoline (no longer common), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), brominated flame retardants and pesticides, many of which may be adhered to the surface of

airborne particulates (Seinfeld, 1989, de Wit, 2002, Guo *et al.*, 2003, Harner *et al.*, 2004, Peverly *et al.*, 2015). However, since several of these compounds are more commonly found indoors than in outdoor air (Wilford *et al.*, 2004), show decreasing trends in urban concentrations in recent decades (Whyatt *et al.*, 2003), and are covered by neither the U.S. EPA, WHO or Canadian ambient air quality legislation, they were not included in the scope of this study.

The choice of contaminants to monitor in Calgary was dictated by three main factors; whether the contaminant i) exhibited local spatial variation, ii) had implications for health, iii) was possible to measure using affordable, reliable passive devices (electricity not consistently available). Consequently, ozone and particulate matter were not measured for reasons i) and iii), respectively, despite having known adverse health effects. In the USA, concentrations of ambient lead have decreased thirty-fold since it was banned from gasoline in the 1970s (U.S. EPA, 2013a), and therefore was not included in this study. Carbon monoxide is discussed briefly in the context of confined spaces, as an Occupational Health & Safety component of experimental planning (Chapters 4 and Appendix D), rather than as a component of ambient air pollution.

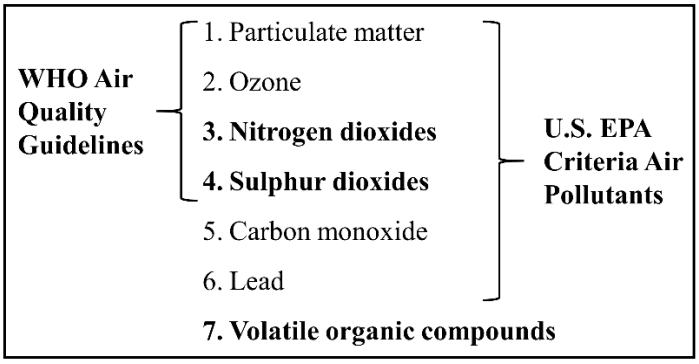

Figure 1-1. Regulated air pollutants. This study focused on the toxicity of the pollutants in bold font.

This section summarizes the most important characteristics of the pollutants relevant to this study (Figure 1-1); more detailed information on these pollutants is available in Curtis *et al.* (2006) and Costa (2008), among others. Regulatory guidelines and Calgary ambient concentrations of several pollutants are listed in Table 1-1.

1.1.4.1 Nitrogen dioxide

In high concentrations, nitrogen dioxide (NO_2) may cause irritation of the mucosal membranes, shortness of breath and ultimately death (Carson, 2004). Common laboratory species like rats, mice, rabbits, guinea pigs and dogs have a mortality threshold of 40–50 ppm for 1-hour. Acute high exposures are more toxic than chronic exposure to low concentrations. High concentrations continue to damage the alveoli post-exposure leading to death.

Ambient NO_2 formed predominately by combustion of fossil fuels for heating, vehicles, and coal-fire power-plants, affects the respiratory system, exacerbating asthma symptoms and contributing to premature mortality (U.S. EPA, 2016a, WHO, 2016). It has been proposed that, rather than being the direct cause of adverse health effects, NO_2 may rather be a surrogate for other co-emitted pollutants (Brook *et al.*, 2007), although this is disputed (U.S. EPA, 2016a). There is major regulatory concern for its role in the formation of ozone in the presence of sunlight, along with volatile organic compounds (U.S. EPA, 2013b, Anon, 2016b, Maas and Grennfelt, 2016).

1.1.4.2 Sulphur dioxide

Sulphur dioxide (SO_2) is produced during the combustion of sulphur-containing fuels, with power plants, smelters, maritime shipping and diesel vehicles being the major anthropogenic sources of SO_2 (U.S. EPA, 2008c, WHO, 2016). Regulations were put in place for SO_2 after confirming its role in the formation of acid rain and subsequent ecosystem effects, damage to building materials, and long-range transport across international borders (Cowling, 1982, Governments of the United States of America and Canada, 1991, Anon, 2015). This water-soluble gas irritates mucus membranes, causing bronchoconstriction and mucus secretion, exacerbating symptoms of bronchitis and asthma (WHO, 2016).

1.1.4.3 Carbon monoxide

Carbon monoxide (CO) is a colourless, odourless, highly-toxic gas, formed when carbon-containing fossil fuels undergo incomplete combustion. Primary sources of CO exposure in urban areas include motor vehicles (most important), occupational exposure, faulty stoves or heaters and

cigarette smoke. Carbon monoxide is fractionally lighter than air, and causes headaches, drowsiness and ultimately asphyxiation (Henderson *et al.*, 1921, Carson, 2004).

Lethal doses vary within and among species (Figure 1-2), with age, metabolic rate, physical activity, and lung/heart function all playing key roles. Animals, usually pets, are frequently affected by CO intoxication sooner and more severely than people sharing their environment (Carson, 2004, Pickrell, 2007). Specific physiological characteristics of birds, such as highly efficient gas exchange mechanisms, metabolic rate and smaller size results in them being unusually sensitive to inhaled toxicants like carbon monoxide, responding more acutely and severely to exposure than mammals (Brown *et al.*, 1997).

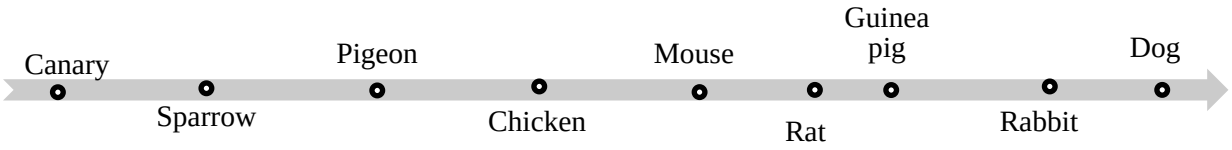

Figure 1-2. Relative sensitivity of different species to carbon monoxide, from most sensitive (left) to least (right) (Brown *et al.*, 1997)

From 1 April–1 May 2014, carbon monoxide levels in central Calgary fluctuated between 0.1–0.4 ppm, with peak values of 0.7–0.9 ppm (CRAZ, 2016). With these peak values being less than a tenth of WHO guidelines, it is evident that ambient carbon monoxide is not of concern in Calgary.

1.1.4.4 Volatile organic compounds

Volatile Organic Compounds (VOCs) are a large group of aromatic hydrocarbons, including many from biological sources associated with familiar scents and tastes. In this study, we focus on anthropogenic, non-methane VOCs known to have adverse health effects at industrial- or ambient concentrations, that are formed during the incomplete combustion of petroleum fuels by vehicles (Inoue *et al.*, 1988, Medinsky *et al.*, 1994, Agency for Toxic Substances and Disease Registry (ATSDR), 2004, Olsgard *et al.*, 2009, Fracasso *et al.*, 2010). At ambient concentrations, regulators' primary concern with VOCs is their role in the formation of ozone, with NO_2 (Anon, 2016b).

Table 1-1. WHO, U.S. EPA and Alberta regulatory thresholds for air contaminants measured in this study (from North *et al.* (2017), Supporting Information Table S4)

Compound	Exposure duration *	WHO Guidelines [a]	U.S. EPA NAAQS [b]		Alberta Ambient Air Quality Objectives [c]	Calgary (mean±SD) [d]
			Primary (public health)	Secondary (public welfare)		
NO_2	1h	200 μg/m^3 (~105 ppbv) †	100 ppbv		300 μg/m^3 (159 ppbv)	4.64 ± 0.24 ppbv
	annual	40 μg/m^3 (~21 ppbv) †	53 ppbv	53 ppbv	45 μg/m^3 (24 ppbv)	
SO_2	10 min	500 μg/m^3 (~188 ppbv) †				0.31 ± 0.03 ppbv
	1h		75 ppbv		450 μg/m^3 (172 ppbv)	
	3h			0.5 ppmv		
	24h	20 μg/m^3 (~7.5 ppbv) †			125 μg/m^3 (48 ppbv)	
	30d				30 μg/m^3 (11 ppbv)	
	annual				20 μg/m^3 (8 ppbv)	
VOCs						
Benzene	1h				30 μg/m^3	0.25 ± 0.08 μg/m^3
	annual				3 μg/m^3	
Toluene	1h				1,880 μg/m^3	0.85 ± 0.36 μg/m^3
	24h				400 μg/m^3	
Ethylbenzene	1h				2,000 μg/m^3	0.14 ± 0.08 μg/m^3
Xylenes	1h				2,300 μg/m^3	0.75 ± 0.35 μg/m^3
	24h				700 μg/m^3	

* Mean concentrations of pollutant over specified period. [a] World Health Organization (WHO) Media center: Ambient (outdoor) air quality and health, fact sheet. Updated September 2016. URL: http://www.who.int/mediacentre/factsheets/fs313/en/. [b] U.S. EPA National Ambient Air Quality Standards (NAAQS), criteria air pollutants. Updated 20 December 2016. URL: https://www.epa.gov/criteria-air-pollutants/naaqs-table%20. [c] Alberta Ambient Air Quality Objectives and Guidelines Summary, document number: AEP, Air Policy, 2016, No. 2. Updated June 2016. ISBN 978-1-4601-2861-9 (PDF). [d] For 2013-2015, from North *et al.* (2017), Chapter 4. † Conversion between μg/m^3 and ppbv (parts per billion, by volume) for NO_2 and SO_2 based on conversion factors published by the UK Air Quality Archive (using the method for reporting data to the European Commission) (Nitrogen dioxide 1 ppbv = 1.9125 μg/m^3; Sulphur dioxide 1 ppbv = 2.6609 μg/m^3)

1.1.5 Calgary region airshed: major pollutants, sources and regulatory priorities

In Canada, particulate matter with aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$) and ozone (O_3) were the first two pollutants to be controlled as part of the Air Quality Management System,

with targeted reduction programs implemented from 2012 (CCME, 2011, Cloet *et al.*, 2011). The PM and ozone management plan was commissioned in Calgary because the city reached trigger concentrations on $PM_{2.5}$ in 2009–2011, and was required by CASA to create a proactive plan to manage these pollutants (SNC-Lavalin, 2014). As ozone precursors, controlling the oxides of nitrogen (NO_x) and VOCs is critical for programs needing to reduce $PM_{2.5}$ and O_3 (SNC-Lavalin, 2014).

In Calgary, light-duty gasoline trucks and cars together contributed 21% of NO_x and 12% of VOC emissions in 2006 (Cloet *et al.*, 2011). In 2008, the transport sector was the largest source of NO_x and VOCs, responsible for almost 79% (NO_x) and 30% (VOCs) of the emissions. Light-duty gasoline vehicles produced 6,849 tonnes of the NO_x and 9 tonnes of VOCs in 2008, responsible for 12.5% and 40.6%, respectively, of the emissions from the transport sector as a whole (Yang *et al.*, 2013).

1.2 Avian Sentinels of Airborne Contaminants

Regarding animals used in ecotoxicology, O'Brien *et al.* (1993) propose definitions to clarify the widely used and interchanged terms, indicator, monitor, and sentinel, suggesting that the latter only be used in the context of species that provide early warning of environmental contamination that may be detrimental to human health, a definition that has become widely accepted (Van der Schalie *et al.*, 1999).While sentinels may include any species (plant, animal, invertebrate, etc.) that serves as an early-warning system for the risk from environmental hazards to humans, in this thesis, the term sentinel is used to refer generally to animals exposed to environmental contamination, as per National Research Council (1991). This definition is broader than that proposed by Beeby (2001), since we feel that sentinels have the capacity to exhibit measurable responses to exposure to a wide range of contaminants, beyond those that bioaccumulate in tissues.

Wildlife, including birds, fish, mammals and amphibians, serve as sentinels of environmental pollution, with mass mortalities, population declines, or clinical signs of ill health warning of possible effects in other animals and people sharing the environment (Colborn *et al.*, 1997). The effects of air pollution on wildlife, particularly after industrial accidents, has been widely noted, serving to enhance public awareness of the toxicological consequences of industrial emissions

(Newman, 1979, Pandey *et al.*, 1986). Domestic animals have also served as sentinels of urban and indoor contaminants (Reif, 2011). However, the use of sentinels to detect early, pre-clinical effects of air pollution is a more recent addition to the ecotoxicological toolbox. The intensification of mining during the 19-20th centuries saw the first documented use of avian sentinels, when canaries, which are more sensitive to the toxic gases than humans, were used to detect dangerous levels of carbon monoxide and methane (Burrell, 1914, National Research Council, 1991, Brown *et al.*, 1997). In her seminal best-seller, Rachel Carson kicked off the global environmental movement, when she reported the effects of pesticides on bird populations in the United States (Carson, 1962). Subsequently, birds have been used as sentinels for monitoring many different environmental contaminants, from marine pollutants (Burger and Gochfeld, 2004), to industrial emissions (Llacuna *et al.*, 1993, Eeva and Yliopisto, 1996) and contaminated sites (Arenal and Halbrook, 1997).

The benefits of using birds as sentinels for studying the ecotoxicology of air pollution are multi-fold. The avian respiratory system has morphological, physiological and mechanical adaptations optimising the efficiency of gas exchange compared with mammalian lungs, both at rest and during exercise, making birds more sensitive to many airborne compounds and therefore early sentinels of inhaled toxicants (Brown *et al.*, 1997). Domestic birds may share the same indoor environment as people and serve as warnings of indoor contaminants like carbon monoxide (Mutluoglu *et al.*, 2012), while many species of wild birds share our urban outdoor environment, potentially providing insight into the effects of contaminants on the health of other urban residents (Drasch *et al.*, 1987, Sicolo *et al.*, 2010, Bókony *et al.*, 2012, Herrera-Dueñas *et al.*, 2014). It is generally easier to get ethics approval to monitor birds than people, it is possible to collect invasive samples that may be sensitive to low-level exposure and thus detect early, subclinical effects, birds may have fewer confounding co-contaminants (e.g., nestlings confined to the nest are exposed to local air pollution), and with their high metabolic rate and shorter lifespans, birds are likely to show adverse effects of exposure sooner than humans (O'Brien *et al.*, 1993). Additionally, there are avian species representing different trophic levels that are globally abundant, common urban residents, with decades of population data available from bird breeding surveys (Carere *et al.*, 2010).

It should be acknowledged that differences between avian and mammalian respiratory systems (e.g., lack of resident pulmonary macrophages and enzymatic differences in birds' lungs) may preclude some avian responses from being closely representative of potential health effects in humans (Brown *et al.*, 1997, Van der Schalie, 1997); however, the information gathered from avian sentinels is undeniably valuable for predicting the effects of exposure on other wildlife, and supporting regulations to protect indigenous wildlife. While evidence of human health effects is the principal driver for public concern, regulatory pressure and theoretically, continued funding, the risk of environmental contaminants is not confined to humans alone, justifying the use of sentinels as part of a broader approach to monitoring shared risk (Rabinowitz *et al.*, 2008).

1.3 The European Starling (*Sturnus vulgaris*)

1.3.1 Natural history

The European starling (*Sturnus vulgaris*), also known as the common starling, is a songbird (passerine) found living successfully alongside humans in urban and rural areas throughout much of the temperate world. Native to the UK, Europe and Western Asia, homesick immigrants introduced them to New York City in the 1890s as a reminder of wildlife they associated with home. Within a century, the starlings had colonized across the continent, to a population size of approximately 200 million birds by the 1950s (Kessel, 1953). Starlings are successful invaders throughout North America from southern Canada through to Central America (Kalmbach and Gabrielson, 1921). They are year-round residents for most of their distribution range and are migratory summer visitors for the most northern areas, including Calgary.

1.3.2 Starlings as sentinels

European starlings meet many criteria for good sentinel species, including those listed by Carere *et al.* (2010): they are locally and regionally abundant invasive species in North America (Kessel, 1953), with large populations closely associated with urban and rural settlements; they readily utilize artificial nest boxes, and tolerate human interference with offspring (Collins and De Vos, 1966, Arenal *et al.*, 2004); and their life-history, ecology, behaviour, and physiology are well-known. As a result, ecotoxicological researchers may monitor large numbers of starling nestlings

in many sites (urban or rural) with varying contaminant burden, and are more likely to receive ethical and wildlife research permission to take invasive samples, which may detect subclinical changes in response to exposure. These factors increase the likelihood of detecting dose-response relationships if they exist.

A diet of terrestrial invertebrates makes starlings sensitive to accumulated, soil-borne contaminants (Arenal *et al.*, 2004), in addition to airborne toxicants. Starlings have been used as sentinels for endocrine disrupting compounds (Markman *et al.*, 2011), heavy metal and PCB contamination (Arenal and Halbrook, 1997), lead contamination near highways (Grue *et al.*, 1986), and in experimental trials investigating the toxicity of pesticides (Grue and Shipley, 1984, Rattner and Grue, 1990, Parker and Goldstein, 2000), methylmercury (Carlson *et al.*, 2014) and cadmium (Congiu *et al.*, 2000), among others.

Wolfe and Kendall (1998) demonstrate that while adult starlings are less sensitive to certain organophosphate pesticides than red-winged blackbirds, for both species, nestlings were significantly more sensitive than adults, possibly due to undeveloped, immature acetylcholinesterase activity.

Starlings have been part of popular history since approximately 1597 (Wikipedia, 2014), when Shakespeare mentioned them in the play King Henry IV (Part 1, Scene 3). Harry Percy (Hotspur), who was angry with the King for refusing to ransom his brother-in-law, suggested that he would train a starling to say the name of Mortimer repeatedly to drive the King mad.

"Nay, I will; that's flat:
He said he would not ransom Mortimer;
Forbad my tongue to speak of Mortimer;
But I will find him when he lies asleep,
And in his ear I'll holla 'Mortimer!'
Nay,
I'll have a starling shall be taught to speak
Nothing but 'Mortimer,' and give it him
To keep his anger still in
motion."(Shakespeare, 1597)

In 1871 a group of New York citizens founded the American Acclimatization Society, with the goal of introducing European flora and fauna to North America. In 1877 the society was chaired by a pharmacist by the name of Eugene Scheiffelin, and per some accounts it was he who decided that for aesthetic reasons they should orchestrate the introduction of all the birds mentioned in Shakespeare's works, including the House Sparrow (*Passer domesticus*) and the European Starling (*Sturnus vulgaris*). Less than 200 years later there were in excess of 200 million starlings throughout the North America (Wikipedia, 2013).

The relative sensitivity of starlings to air pollution is not known, however, the many benefits of using this species rather than a less-prolific, native species justify the use of European starlings in this investigation into the effects of exposure to ambient, urban air contamination.

1.4 Biomarkers of Ecotoxicity

Descriptions of the methods used to measure these biomarkers may be found in the main text, with reproductive and nestling-related responses detailed in Chapter 3 and Appendix C, and biochemical responses in Chapters 3 and 4. The biomarkers of interest for this study are listed in Table 1-2.

Table 1-2. Biomarkers evaluated for sensitivity to exposure to urban air pollution, in European starlings

Parent birds	**Nestlings & Adults (Experiment)**			
Reproduction	**Growth & development**	**Endocrine & immune responses**	**Biochemical responses**	**Histopathology**
Clutch size	Tarsal length	Cell-mediated immunity [3]	Hepatic detoxification [5]	Thyroids
Egg mass	Wing chord	Thyroid hormones [4]	Oxidative stress [6]	
Hatching success	Condition [1]			
Nest success	Organ mass [2]			

[1] Scaled mass index (SMI, Peig and Green (2009)). [2] Size-corrected, scaled organ mass indices (Rodríguez-Estival *et al.*, 2015). [3] Using phytohaemagglutinin skin test (PHA, Smits *et al.* (1999)). [4] Free and total thyroxine (fT4 and TT4), total triiodothyronine (TT3). [5] 7-ethoxyresorufin-*O*-deethylase (EROD). [6] Glutathione (GSH), oxidized glutathione, or glutathione disulfide (GSSG), thiobarbituric acid reactive substances (TBARS).

1.5 Confounding Factors

All field studies are affected to some degree by confounding influences (Table 1-3). These should be limited as far as possible by the study design, for example, by having only one investigator take all measurements or by visiting the nests at the same time each day. For uncontrollable confounders like hatch date, weather, parent quality or diet, these should be acknowledged and incorporated into statistical analyses. Factors that cannot be accounted for should be minimized, and should be clearly discussed in relation to results. Several important possible confounders are discussed in Chapters 3 and Appendices B and C.

While experimental studies attempt to control confounders, some are still relevant.

Table 1-3. Confounding factors in field and experimental toxicology

	Field study	Experimental study
Controllable	Investigator bias	Investigator bias
	Measurement error	Measurement error
		Sex-linked differences
		Different level of disturbance
		Temperature & humidity
Uncontrollable	Weather*	Genetics
	Parent quality	Previous exposure
	Unmeasured co-contaminants	
	Diet	
	Genetic differences	
	Inter-annual differences in lab methods*	

* These are measurable or the data are publicly available

Unmeasured co-contaminants include: noise and light pollution, local, approved pesticide application, and contaminated sites from prior industrial use. These are discussed in Chapter 5.

1.6 Research Objectives

Regarding common air pollutants:

1. To establish reliable, affordable methods of determining concentration and distribution of air contaminants,
2. To identify sensitive biomarkers of exposure to common urban air contaminants, that are practical for use in wild birds and applicable in field and experimental studies,
3. To evaluate the sensitivity of European starlings as sentinels for health effects of ambient air pollution, based on the biomarkers examined in natural and experimental exposures.

How these objectives have been met will be discussed in the chapters that follow.

Chapter Two: **Validation of passive samplers for measuring air pollution in ecotoxicology studies**

2.1 Introduction

Air pollution is of major concern among medical health professionals globally, as an increasing number of epidemiological studies provide evidence supporting a causal link between exposure to air pollutants and health effects like diabetes, asthma, and other cardiorespiratory disease (Sunyer *et al.*, 2003, Patel *et al.*, 2010, Chen *et al.*, 2013). The World Health Organisation stated that in 2012 alone, outdoor air pollution caused 3.7 million premature mortalities globally (WHO, 2016), leading the organisation to demand remedial action by cities and policy-makers to ensure they meet published air quality guidelines.

Monitoring agencies and consulting firms have expended immeasurable time and money characterizing, modelling and predicting 'plume' dispersal of contaminants coming from point- and line-sources, and the atmospheric chemistry and long-range transport that determines regional and global pollution burdens. However, the technology and specialized skills required to work with these data are beyond most toxicologists, to whom the physiological and biochemical responses of the species being studied are of greatest interest. Aside from technological and computing specialization, the costs involved using state-of-the-art, sensitive, accurate and 'real-time' (RT) or 'near-RT' equipment is prohibitive to most researchers.

The use of personal, passive-type air samplers is a cost-effective and versatile solution for many diverse research projects (Roadman *et al.*, 2003, Mukerjee *et al.*, 2004, Jerrett *et al.*, 2009). The gas concentration measured using passive samplers provides the concentration of that pollutant per minute of exposure, based upon an integrated, average concentration for the period of deployment. Passive samplers typically have minimum and maximum recommended deployment durations based on the expected concentrations. The samplers do not require electricity to run, enabling sampling in diverse sites including secluded, rural locations; however, the samplers need to be sheltered from adverse weather.

Many studies estimate exposure based on distance-from-source (Llacuna *et al.*, 1993, Schilderman *et al.*, 1997), or use air quality data obtained from fixed, central monitoring stations to study the

effects of exposure to air pollution on the health of residents (Braun-Fahrlander *et al.*, 1996, Ackermann-Liebrich *et al.*, 1997, Samet *et al.*, 2000, McGowan *et al.*, 2002). While this offers a useful approximation of exposure at a population level and matches the postal code or county-level collection of health data, it ignores differential exposure of individuals resulting from local variation in pollution and their personal movements around the city, obscuring possible related impacts on individuals' health. Local, spatial differences in air quality may be influenced by factors such as topography and prevailing wind direction, as well as proximity of pollutant sources like major roads or industry.

Passive samplers provide a cost-effective alternative, measuring cumulative exposures, although compromising the temporal precision by integrating amount of pollutant exposure over the duration the samplers were deployed. Another important benefit of passive samplers is the minimal infrastructure required; no electrical supply is necessary, and samplers may be deployed in public areas without notice. Several regional monitoring networks like the Calgary Region Airshed Zone (CRAZ), deploy passive samplers to complement central monitoring sites, thus extending the total area monitored without sacrificing temporal precision in the most densely occupied parts of the city.

There are many types of passive samplers for monitoring air quality, some available commercially and others specifically designed by researchers. Mosses and lichens have been used to assess air pollution for decades through their presence/absence (LeBlanc *et al.*, 1972), and more recently by pollutants passively adsorbed to the lichen from the environment (Conti and Cecchetti, 2001, Szczepaniak and Biziuk, 2003). For the purposes of this study, however, commercially-available, validated passive samplers for measuring nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and volatile organic compounds (VOCs) were chosen for ease of use under field conditions.

Land-Use Regression (LUR) methods involve statistical models predicting air quality based on the proximity of nearby land-use types, for example major roads, residential and industrial areas, and may incorporate weather, wind direction and topographic predictors (Su *et al.*, 2008). These models provide valuable exposure data for epidemiological studies, and can be used retrospectively if the land-use data are available. Different models, with different labels (e.g.,

geographically-weighted regression) may include different predictors, and have variable specificity, complexity and objectives (Ryan and LeMasters, 2007, Bertazzon *et al.*, 2015).

Many Canadian and U.S. cities have one or more fixed central monitoring stations monitoring a suite of pollutants important for calculating an Air Quality Health Index, in real-time or near real time. These monitors are usually operated and maintained by professional technical staff, and generate high-quality data. While these may be regarded as gold-standard methods, the data must be extrapolated to estimate population exposure, which misses local variation in air quality. Precision decreases with increased distance from the monitor (Marshall *et al.*, 2008, U.S. EPA, 2016a), reducing the sensitivity of epidemiological studies considering health effects.

There have been a few studies comparing the concentrations measured by passive samplers, including those used in our work, in field settings to active monitors (Mukerjee *et al.*, 2004). However, there have been no studies comparing field deployed passive samplers to land-use regression predictions and fixed central monitoring station measurements for the same region and season. We feel that these comparisons will improve the confidence and insight into other studies using these different methods to estimate exposure to air contaminants.

While in this chapter we compare the pollution concentrations measured using passive monitors to those predicted using land-use regression, the two methods are not mutually exclusive, since LUR is frequently developed using data from passive monitors (Oiamo *et al.*, 2015). Characteristics of the three methods are summarized in Table 2-1.

Table 2-1. Characteristics of central (fixed) monitors, LUR models and passive samplers for use in toxicology studies

	Fixed monitoring stations	Land-use regression (LUR) models	Passive samplers
Initial, set-up cost	High	Moderate [a]	Moderate-low
Ongoing cost [b]	Moderate-high	Low	Moderate
Required infrastructure	High	Low	Low
Technical expertise	High	High	Low
Temporal resolution	High	Moderate	Moderate-low
Spatial resolution	Low	Moderate-high [c]	High [d]
Validation	Daily internal positive & negative control 'spans'	Comparison of model predictions to existing monitoring data	Field and trip blanks, duplicates, routine laboratory QA/QC methods

[a] Requires high-quality data to start with. [b] Including analysis, replacement parts, etc. [c] Depends on quality of original data. [d] Depends on the density of passive sampler deployment.

2.2 Materials and methods

2.2.1 Study sites and passive samplers

As part of a field study on air pollution toxicology, air pollution concentrations were measured using passive samplers in ten urban parks in the city of Calgary, over a three-week period in late May–June for 2013–2015. Sites were selected to include areas with low and high traffic density that were also suitable as nest sites as part of an avian toxicology study (North *et al.*, 2017). Variation in pollution levels during the summer were assessed by deploying a second set of samplers in two locations during July 2014 and 2015. Study site abbreviations and regions are listed in Table 2-2.

Integrated concentrations for the monitoring periods were obtained for nitrogen dioxide (NO_2) and volatile organic compounds (VOCs, including benzene, toluene, ethylbenzene and xylenes, integrated as BTEX) using passive, personal-type samplers (NO_2, Ogawa USA, Pompano Beach, Florida, and VOCs 3M^{TM} Organic Vapor Monitor 3500, USA). At least one trip blank was run for each sampler type each year, and in 2015 two sites had duplicate samplers. Samplers were handled

per manufacturer instructions, protected from precipitation, and were analysed by the Alberta Centre for Toxicology according to manufacturers' guidelines (Appendix A).

Table 2-2. Regions, sites (referred to as 'locations') and abbreviations used for this study

Region	Location	Abbreviation
0 (reference)	**Hull's Wood**	HW
	Deer Run	DR
1	**Montgomery**	MG
	Woods Homes	WH
2	River Park	RP
	Sandy Beach	SB
3	**Hidden Valley**	HV
4	**Pearce Estate**	PE
	Inglewood	IN
	Marlborough	MB

2.2.2 Comparative data

To validate the passive sampler data and the pollutant distribution (high versus low areas) in the city of Calgary, two sources of air pollution data were used for comparison: i) summer (July-August) land-use regression model data for each site (assessing spatial variation within the city), and ii) the Calgary Region Airshed Zone's three, fixed monitoring stations (temporal variation, between early and late season and among years, Figure 2-1).

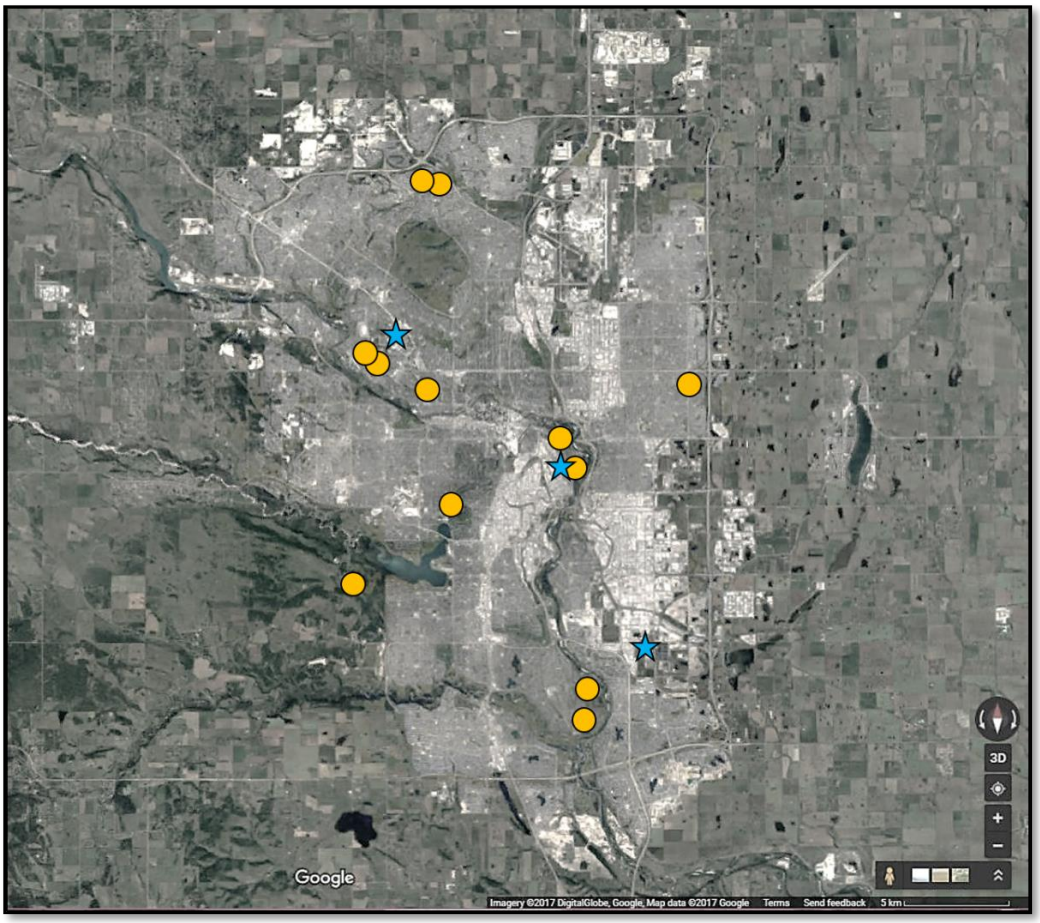

Figure 2-1. Passive sampling sites for 2013–2015 (yellow circles) and CRAZ fixed continuous monitoring sites (blue stars) in Calgary, AB (only the central and northwest sites were used for these comparisons). Overlapping circles represent samplers deployed within the same park (200–800 m apart). Not all the sites were measured in all years.

2.2.2.1 Land-use regression data

Health Canada, together with researchers from the University of Calgary, measured air quality at approximately 50 sites in summer and the winter of 2010/2011, testing which land-use factors best predicted pollution levels to develop preliminary LUR models (Bertazzon *et al.*, 2015). The study was repeated in 2015/2016, covering a greater area with 125 monitoring sites and increasing the number of pollutants measured. These data have been used to test and refine the original LUR models, and assess temporal (monthly, seasonal and annual) variability in exposure to these pollutants (Johnson *et al.*, 2016). Summer NO_2 (2010), plus NO_2 and BTEX (2015) LUR values were compared with concentrations from the passive samplers in 23 sites in 2013–2015.

2.2.2.2 Fixed monitoring stations

To compare the passive samplers with the fixed, central monitoring stations operated by CRAZ during the same period, hourly air quality data were extracted for May–August of 2013–2015 from Alberta Environment and Parks archived air quality data (http://airdata.alberta.ca/). Only NO_2 data from the northwest and central stations were available for comparison with the passive samplers. The hourly data for the two stations were averaged for the passive monitoring periods for each year (25 May–15 June in 2013, 23 May–13 June and 2–23 July in 2014, 18 May–9 June and 24 July–7 August in 2015).

Volatile organic compound concentrations were only available from the National Air Pollution Surveillance (NAPS) database (http://maps-cartes.ec.gc.ca/rnspa-naps/data.aspx?lang=en); the data are integrated over a six-day collection period. The only station in Calgary measuring the speciated VOCs is the downtown station (Calgary Central, managed by CRAZ). Fixed monitoring data could therefore not be used to assess annual variability in BTEX.

Comparisons between the three methods measurement dates and equipment is given in Table 2-3.

Table 2-3. Details of the dates and technology measuring nitrogen dioxide (NO_2) and BTEX for the three methods

	Fixed monitoring stations	**LUR models (summer)**	**Passive samplers**
Sampling dates	2013 25 May – 15 June 2014 23 May – 13 June 2 July – 23 July 2015 18 May – 9 June 24 July – 7 Aug	2010 4–18 Aug 2015 5–19 Aug	2013 25 May – 15 June 2014 23 May – 13 June 2 July – 23 July 2015 18 May – 9 June 24 July – 7 Aug
Monitoring equipment	NO_X: TEI 42C (NW, until May 2015) TEI 42i (Central & NW from 2015)	NO_2: Ogawa [a] BTEX: 3M OVM [b]	NO_2: Ogawa [a] BTEX: 3M OVM [b]
Data type	RT, [hourly]	Original data: [minute] 2-wk deployment Statistical models: Land-use ± wind ± season ± topography = predicted air quality	[minute] 3-wk deployment

[a] Ogawa USA, Pompano Beach, Florida. [b] 3MTM Organic Vapor Monitor 3500, USA. RT = real-time, [hourly] and [minute] = averaged concentrations over specified time, wk = week

2.2.3 Statistical analysis

The relative ranking of different areas for NO_2 concentration by passive sampler and LUR (2010 and 2015) was assessed using Spearman's correlation (i.e., "dirty" areas consistently dirty among methods, "clean" areas consistently clean). The difference between early and late summer, and among years, was assessed for the fixed monitoring station data using students' t-test and one-way Analysis of Variance (ANOVA) with LSD, respectively. Passive data were not analysed for season (small sample size); the difference between years was analysed using non-parametric, independent-samples median- and Kruskal-Wallis tests. Analyses were conducted using SPSS statistical software version 24.0 (IBM SPSS Inc., Chicago, IL, USA), with two-tailed significance set to $p<0.05$.

2.3 Results and Discussion

LUR 2015 NO_2 values were abnormally distributed, with a cluster of high concentrations (20 ppb) at one site. These were not removed, however, since they represent the output of the model, and are of interest for the comparison with passive samplers. The data exported from CRAZ had to be converted from ppm to ppb concentrations. There were a few missing values (usually during the instruments' daily calibration 'span' periods or during maintenance).

2.3.1 Summer ambient air pollution

Median concentrations of NO_2 and BTEX in Calgary, Alberta during May–August of 2013–2015 were 4.8 ppb and 1.8 µg/m^3 using passive samplers, and 4.6 ppb and 1.6 µg/m^3 from 2015 LUR prediction models, respectively. During the same period, NO_2 concentrations were 8.3 and 4.8 ppb at CRAZ fixed monitoring sites (central and northwest, respectively, Table 2-4).

Table 2-4. Nitrogen dioxide (NO_2) and BTEX in Calgary, measured using three methods

	NO_2 (ppb)					BTEX (µg/m^3)	
	Passives [a]	**LUR 2010 [b]**	**LUR 2015 [c]**	**Central [d]**	**NW [d]**	**Passives [a]**	**LUR 2015 [c]**
n[*]	23	23	23	2419	2468	23	23
n (true)[**]	11	11	11	n/a	n/a	11	11
Mean ± SD	4.8 ± 1.8	11.1 ± 2.5	7.9 ± 6.2	9.6 ± 6.3	5.8 ± 4.1	1.9 ± 0.7	1.7 ± 0.4
Median	4.8	12.6	4.6	8.3	4.8	1.8	1.6
Range	2.1–8.4	6.6–13.4	3.4–20.0	0.0–38.5	0.5–38.0	0.8–3.0	1.2–2.3

[a] Measured using passive samplers during May–August of 2013–2015. [b] Based on measurements from August 2010. [c] Based on measurements from August 2015. [d] Averaged hourly data from central (downtown) and northwest (NW) fixed monitoring stations during May–August 2013–2015. [*] Number of discrete data points. [**]True sample size refers to the number of separate sites that were sampled multiple years. Mean ± standard deviation (SD).

2.3.2 Passive sampler and land use regression results

Nitrogen dioxide concentrations as measured using passives and predicted by LUR using 2015 data were closely correlated (r_S=0.869, p<0.0001), with a weaker, but still significant correlation with LUR using 2010 data (r_S=0.502, p=0.015). It is worth noting, that while the latter correlation is significant (i.e., the two methods would rank sites similarly), the actual values predicted by the

2010 LUR were considerably higher than the measured concentrations. For BTEX, passive samplers were significantly correlated with LUR 2015 (r_S=0.550, p=0.006). Figure 2-2 shows the relationships between passive and LUR concentrations.

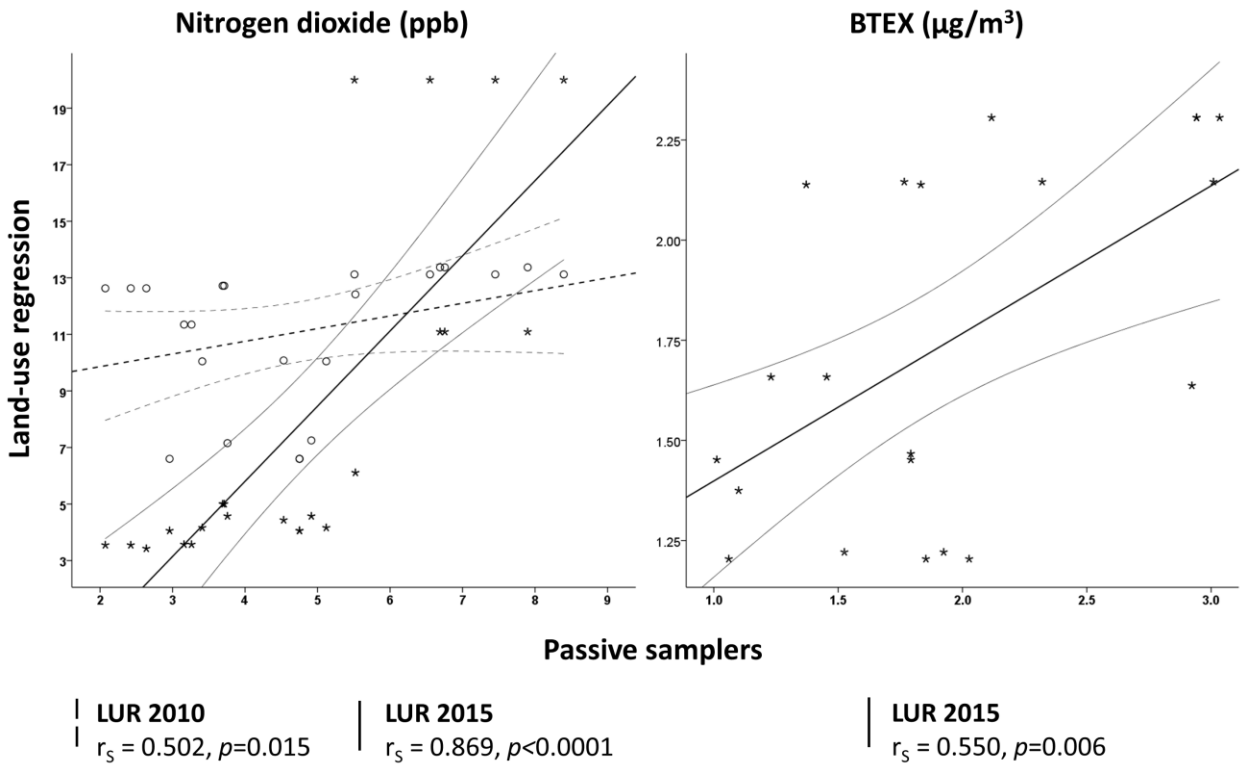

Figure 2-2. Scatterplot of passive sampler data by 2010 and 2015 LUR model predictions. Thin, curved lines represent the confidence interval of the mean.

The passive and consequently, spatially-related LUR data are clustered in seven regions, with only 11 separate monitoring sites that were measured repeatedly during 2013–2015 (Figure 2-3). Thus, analysis should ideally account for repeated measures, however, the limited sample size precludes such methods. It is evident from the figure that LUR values generally agree with passive sampler categorization of regions as relatively dirty or clean.

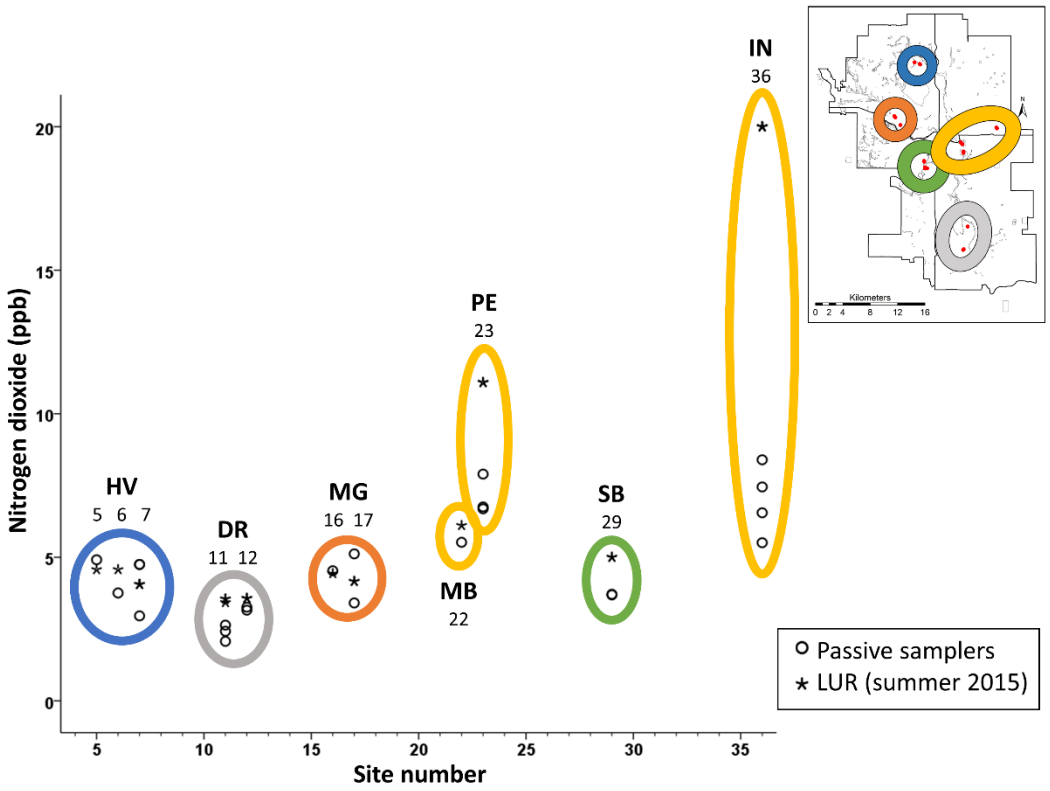

Figure 2-3. Passive sampler and LUR nitrogen dioxide concentrations according to the site of measurement. Colours correspond with Figure 4.4 (inset map from North et al. (2017)). The acronyms (e.g., HV, MG) represent separate urban parks, with the numbers representing individual sampling sites.

2.3.3 Passive sampler and fixed monitoring results

NO_2 concentrations measured at CRAZ Central and NW fixed monitoring stations varied significantly by year ($F_{2,2416}$=18.80, p<0.0001 and $F_{2,2465}$=53.03, p<0.0001, respectively), with the downtown station (Central) having the highest pollution levels in 2014 and the northwest station (NW) its lowest concentrations that same year (Figure 2-4). NO_2 and BTEX concentrations measured using passive samplers were not different among years. If there is year-to-year variation in pollution levels, the passive data are likely not sensitive enough to detect the difference because they include measurements from around the city, resulting in large variability within each year.

Fixed, continuous monitors measured higher concentrations of NO_2 than the passive samplers, with moderately higher average concentrations at both sites. This corresponds with findings by Bytnerowicz *et al.* (2002b), and contrasts with the up to 30% overestimation on NO_2

concentrations by these passive samplers as a result of the reaction between O_3 and NO (Cox, 2003).

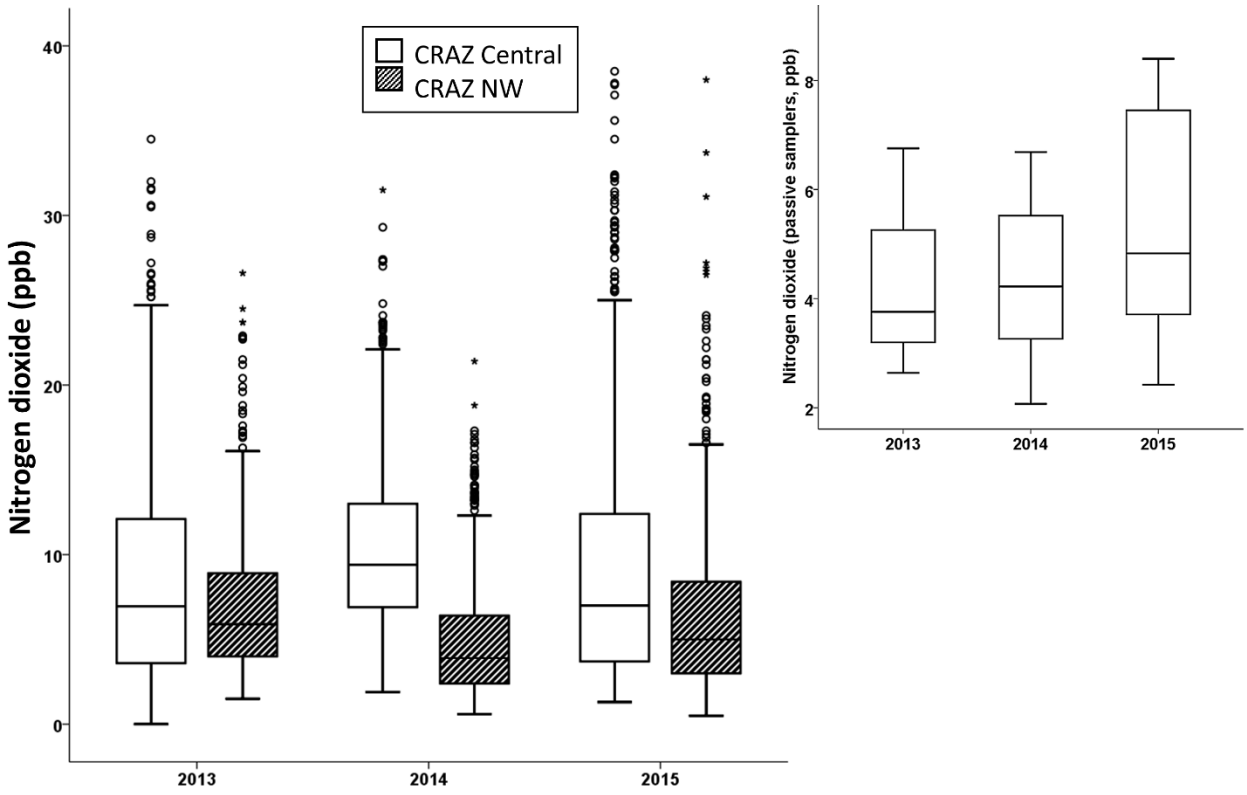

Figure 2-4. Annual and geographic variation in NO_2 concentrations for fixed monitoring sites. Inset: NO_2 concentrations measured by this study using passive samplers, by year.

While there are not enough sampling points to run statistics on the passive sampler concentrations for early versus late summer (n=20 and 3, respectively), both fixed monitoring stations measured higher NO_2 levels early compared to late summer (Central: F_{2417}=37.96, p<0.0001 and NW: F_{2466}=34.63, p<0.0001, Table 2-5). However, the real difference in concentrations is very small (<1 ppb) and likely not biologically important, so it may only be significant because of the large dataset (>2400 points).

Table 2-5. Early and late summer NO_2 concentrations from fixed monitoring stations

	Season	n	Mean ± SEM
CRAZ Central	Early	1578	9.85 ± 0.17
	Late	841	9.25 ± 0.18
CRAZ NW	Early	1592	6.16 ± 0.11
	Late	876	5.27 ± 0.11

n = number of hourly measurements reported

2.4 Conclusions

While there are differences in the absolute concentrations of pollutants measured using passives, the central monitoring sites, and those predicted by LUR models, they are all within approximately 5 ppb. We show particularly, good spatial agreement between passive and LUR (2015) methods.

The model created using NO_2 concentrations measured during the summer of 2015 matches passive sampler values very closely. This spatial agreement between the two methods is valuable for toxicological studies like that of North *et al.* (2017), since contaminant exposure is determined by location in the city. The older, 2010 LUR model has very poor fit with the passive data, both by site and absolute value, with predicted concentrations being more than double those measured. This may be explained by the initial model being based on a smaller dataset with its predictors being less well fitted. Another explanation may be because the initial model used data from 2010 rather than 2015, while the passive samplers were deployed in 2013–2015. Since LUR air pollution predictions are based on land use, they should be able to accommodate urban changes over time.

The passive sampler BTEX concentrations were not as well correlated with LUR, possibly because the models do not include gas stations—a known source of BTEX emissions—as predictors, and several passive sites were within 500 m of a gas station.

There have been many studies on the effects of air pollution in wildlife, although predominately industrial point-source pollution such as one would find near a smelter, refinery or coal-fire power plant. Examples include the effects of airborne heavy metals from factories on two hole-nesting passerines (Eeva *et al.*, 1994, Eeva and Lehikoinen, 1995, Eeva and Lehikoinen, 1996, Eeva *et al.*, 2000, Eeva *et al.*, 2005, Eeva *et al.*, 2009), and a classic multi-species study comparing the effect

of proximity to a coal-fired power plant in two small rodents and three songbird species (Llacuna *et al.*, 1993). Few of these studies, however, measure the concentrations of the air contaminants at their study sites, typically relying on regional measures of pollution provided by state regulators, industrial emissions reports, or on distance from the point-source pollutant being inversely related to concentration.

When deciding on a method for air pollution measurement, it is essential to consider:

- The purpose to which it is being used, i.e., the objectives of the study
- Budget available
- Point-source or dispersed regional pollutants
- Topography and predominant wind patterns

It is obviously always tempting to have the best possible data available for a project, however, sometimes excessively high-resolution data can be misleading, making interpretation of the results harder than when using integrated readings. Using complementary methods of measuring air quality is always the best and most defensible choice.

Personal, passive-type air samplers are a cost-effective and versatile solution for many research projects.

2.5 Pros and Cons

These passive samplers have been proven (by this and other studies) to perform well under field conditions, and are suitable for use in wildlife ecotoxicological studies. The samplers are affordable, versatile, easy to use, and the integrated concentrations more appropriate for assessing the effects of chronic exposure to ambient pollution than peak levels would be, simplifying data analysis.

There are several limitations of the methods used to ‘calibrate’ the passive samplers, that, if not substantiated by numerous published validation of these samplers, would necessitate further efforts. These include the following:

- Passive sampler validation ideally requires the co-location of passive samplers with active monitors to determine the passive sampling rate; unfortunately, this was not done in this study. However, the passive samplers used in this study are commercially available and have been formally validated by other field studies.
- The LUR was based upon air quality measurements using the exact same passive samplers as were used by the authors,
- There was pseudoreplication, with repeated measurements at each sampling site (year-to-year), thus artificially enhancing the sample size. Ideally, if there were more data available, the statistical methods selected could account for this by examining each sampling site as the subject, with the repeated measurements (years) included as fixed factors.
- It was not possible to determine whether passive samplers could detect a seasonal trend in pollution levels, since there were limited data points for the late summer, two of the three being from the same site over two years (a relatively dirty site).

Future studies should ensure that during method validation, passive samplers are co-located with an active monitor (e.g., the CRAZ fixed monitoring sites) to verify passive sampling rate. Additionally, to verify within-season variation, there should be approximately equal number of sites measured both early and late summer. Where possible, it is preferable to incorporate several different, complementary methods of measuring exposure, such as passive samplers and LUR.

Chapter Three: **European starlings (*Sturnus vulgaris*) as sentinels of urban air pollution: a comprehensive approach from non-invasive to post mortem investigation**

3.1 Abstract

Urban, traffic-related air pollution remains a concern to health-care and environmental professionals, with mounting evidence connecting diverse disease conditions with exposure. Wildlife species such as European starlings (*Sturnus vulgaris*) cohabit urban neighborhoods and may serve as sentinels for these contaminants. In this novel approach, we use passive, personal-type air samplers to provide site-specific measurements of nitrogen dioxide (NO_2), sulfur dioxide (SO_2) and volatile organic compounds (VOCs, such as benzene, toluene, ethylbenzene and xylenes, or BTEX), and account for the effects of confounding environmental factors when teasing out the responses to exposure. This study examines biomarkers of exposure to predominately traffic-related, urban air contaminants in European starlings, including morphometric measurements, immunotoxicology, oxidative stress and hepatic detoxification, and analyses responses in the context of multi-layered factors including year, hatch date, weather and location, confirming that this experimental approach and the selected health indicators can be used for comparing locations with different levels of contaminants.

3.2 Introduction

Air pollution has been linked to human illness for centuries (Chen *et al.*, 2007). Epidemiological studies provide evidence linking exposure to air pollution with a range of health effects, for example, nitrogen dioxide, ozone, sulfur dioxide, and asthma (D'Amato *et al.*, 2002, Parnia *et al.*, 2002, Sunyer *et al.*, 2003, Lin *et al.*, 2004); particulate matter with diverse health impacts including diabetes (Chen *et al.*, 2013), autoimmune rheumatic diseases (SARDs) (Bernatsky *et al.*, 2015) and higher mortality from all causes (Samet *et al.*, 2000). Yet, despite these many large-scale studies, consequences of long-term exposure to naturally-occurring mixtures of pollutants is largely unknown.

Burger and Gochfeld (1999) propose using biological indicators to evaluate the health of ecosystems and their natural populations. A sentinel species is a bioindicator used to monitor the

effects of contamination, with the assumption of corrective action if detrimental changes were detected. Biomarkers can include molecular, biochemical, physiological or behavioral responses to toxicants, providing evidence of toxicity to investigators monitoring effects on health of individuals or populations (Ricketts *et al.*, 2004). Examining biological effects of contaminants on sentinel species has become a well-accepted practice, with examples including aquatic invertebrates and fish (Ferreira *et al.*, 2004), mammals (Basu *et al.*, 2007, Bossart, 2011), and marine birds (Burger and Gochfeld, 2004). However, studies using sentinels have almost exclusively focused on water-borne contaminants, with very few examples of wildlife being used to study the effects of air pollution. A biological sentinel offers a true measure of toxic response, whereas simply measuring the concentrations of chemicals in environmental media tells us little of toxicity, and means even less for mixtures as occur in natural conditions (Shugart *et al.*, 1992). Birds, with their unique respiratory system and high metabolic rate, are particularly sensitive to exposure to airborne contaminants (Brown *et al.*, 1997). Additionally, many birds are urban residents with lifelong exposure to air contaminants commonly associated with cities, making them excellent for biomonitoring effects of pollutants. While this is not intended to reflect toxic interactions and responses in humans, the information generated can indicate 'areas of concern' and help prioritize efforts regarding human health risks.

European starlings (*Sturnus vulgaris*) are a highly successful, invasive passerine species throughout much of the world including North America (Kalmbach, 1928, Kessel, 1953). Starlings successfully cohabit with people in many urban areas, sharing our airspace and any contaminants associated with it. They readily colonize artificial nest boxes and tolerate human interference, permitting regular handling of the offspring without causing nest abandonment by the parents (Collins and De Vos, 1966). Starlings have been used as sentinels of environmental contamination in several studies (Arenal *et al.*, 2004, Markman *et al.*, 2011).

Responses of wildlife to toxicants ranges from population-level morbidity/mortality after acute, high exposures from industrial spills (Newman, 1979), to subclinical effects including impaired immune function, behavioral changes, compromised reproduction, and reduced resilience to additional stressors (Gentes *et al.*, 2006, Gentes *et al.*, 2007a). To preserve wildlife species and ecosystem diversity, it is important to detect early indicators of contaminant related stress such as compromised reproductive success, poor growth or survival of offspring, or evidence of

physiological adaptation or compensation to exposure. This study assesses various subclinical, physiological responses for their potential as biomarkers of exposure to air pollution.

Many ground-breaking studies investigating the effects of air pollutants on wildlife have used a central monitoring station to estimate exposure (Llacuna *et al.*, 1993, Eeva and Lehikoinen, 1995). Monitoring air pollution can be extremely costly, requiring electricity, expensive equipment, and technical monitoring and maintenance to obtain reliable data. Personal, passive samplers measuring air pollutants could provide a cost-effective, easy-to-use alternative to traditional methods, integrating pollutant concentration into average minute-values for the period of deployment (1–4 weeks). The use of such samplers has been very limited in ecotoxicological field studies. Their potential to provide site-specific, accurate field-exposure data has been explored in several studies (Roadman *et al.*, 2003, Jerrett *et al.*, 2009, Cruz-Martinez *et al.*, 2015a). The same VOC and NO_2 passive samplers used in this study, were validated by Mukerjee *et al.* (2004).

This study has two primary objectives: firstly, to identify minimally invasive, practical, physiological responses that are sensitive to air pollution in European starlings, and secondly, to complement and compare these with biomarkers requiring lethal sampling. To achieve these objectives, we first need to prove that outdoor air quality (i.e., nitrogen dioxide (NO_2), sulfur dioxide (SO_2) and volatile organic compounds (VOCs), measured using passive samplers) shows spatial variability within the city. Second, conspicuous potential confounding factors need to be identified and tested for significance where possible.

3.3 Materials and Methods

3.3.1 Site selection, pollutants and exposure measurement

Sites providing suitable habitat for wild birds were chosen to include regions of relatively higher and lower air pollution concentrations according to a Health Canada study in 2010/2011 (Bertazzon *et al.*, 2015). Wooden nest boxes (Figure 3-1) were attached to trees and utility poles 3–4 m above the ground in urban parks in Calgary.

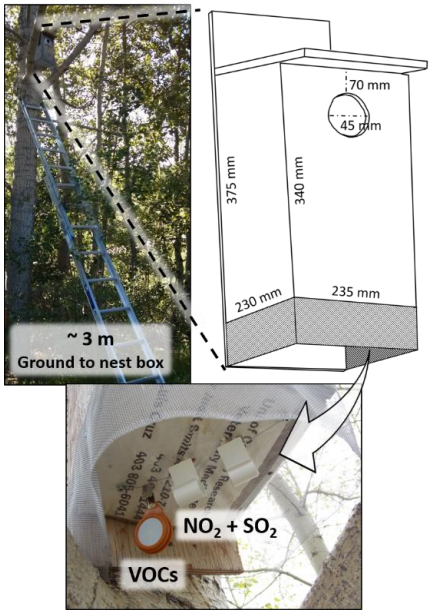

Figure 3-1. Nest box dimensions, passive monitor attachment and rain protection (wire mesh around the base of the nest box).

All parks had similar habitat with grasses, deciduous (balsam poplar and aspen) and coniferous (spruce) trees. The geographic terminology used is explained in Figure 3-2.

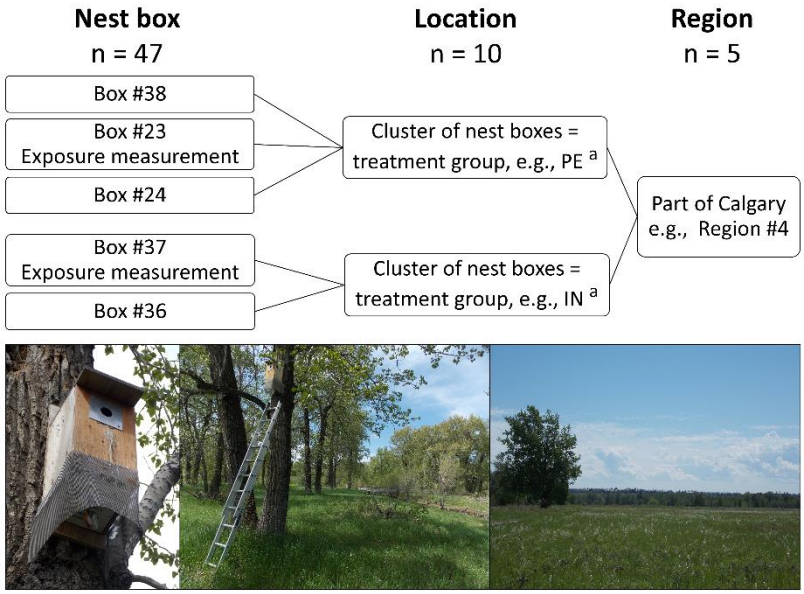

Figure 3-2. Visual portrayal of terminology used to describe the geographic units and grouping, using one of our monitoring regions as an example. Examples of the uniquely numbered nest boxes, which serve as the experimental unit for this study, are shown in the figure (e.g., Box #38). [a] Location names and abbreviations as listed in Table 3-1.

Boxes in one location were all within 1 km of exposure measurements using passive samplers. Locations are summarized in Table 3-1. Locations were grouped into five regions by proximity (e.g., MG and WH), whether they were part of a contiguous urban park with similar environment (e.g., HW and DR, RP and SB), or were discrete regions found to have consistently higher pollution burdens than the rest (e.g., PE, IN and MB).

Which contaminants to monitor was dictated by three factors:

- Whether the contaminant exhibits local spatial variation (e.g., ozone, a regional contaminant, varies little within a city),
- Implications or known health concerns,
- Availability of affordable, reliable passive monitoring devices (electricity not consistently available).

While particulate matter (e.g., $PM_{2.5}$) is a known pollutant of concern, the lack of electricity at sites precluded its measurement in this study. Publicly accessible monitoring data are only available for three sites in the city of Calgary and do not provide the spatial resolution required for this study.

Table 3-1. Regions within the city ranked from 0–4 with increasing pollution concentration, locations with abbreviations used in this paper, and the number of active nests in 2013–2015.

Region	Location	Abbreviation	# Nests [a]		
			2013	2014	2015
0 (reference)	**Hull's Wood**	HW	1	1	0
	Deer Run	DR		2 (1)	2
1	**Montgomery**	MG	1	2	2 (1)
	Woods Homes	WH		1	1
2	River Park	RP		2	2
	Sandy Beach	SB		3	1
3	**Hidden Valley**	HV	1	2 (1)	2
4	**Pearce Estate**	PE	1	3	2
	Inglewood	IN		2 (2)	2 (1)
	Marlborough	MB		2	0

[a] Number of nests: first broods (second broods). Locations with passive samplers in bold font. Second broods were not recorded in 2013.

Passive, personal-type samplers were attached to the bottom of a central nest box in each location, with wire mesh offering protection from rain (Figure 3-1). The samplers were deployed for three weeks between hatching and fledging of the birds during 2013, 2014 and 2015. Volatile organic compounds (VOCs, including benzene, toluene, ethylbenzene and xylenes, collectively referred to as BTEX, see Table B-3; 3M™ Organic Vapor Monitor 3500, USA), NO_2, SO_2 and ozone (Ogawa USA, Pompano Beach, Florida) were measured at each site. Preliminary sampling in 2012 revealed ozone to have no spatial variation within the city, and therefore was not monitored subsequently. Passive air samplers were analyzed according to manufacturers' instructions by the Alberta Centre for Toxicology (Appendix A).

3.3.2 Study area and general methods

The field study was conducted during May to August of 2013 to 2015, in Calgary, Alberta, Canada. In southern Alberta, starlings are migratory, arriving in February–March for the breeding season (May to July), and departing again by October. At this latitude, most pairs raise a single brood with a few second (or late) broods observed in July or August. Adult starling health and quality was measured using reproductive endpoints, while offspring health and development was assessed using a variety of morphometric, immunological and biochemical biomarkers.

Boxes were monitored every 2 to 3 days from the beginning of May to determine nest-building, clutch initiation, clutch size, and anticipated hatching dates (date of penultimate egg laid + 13 d). Eggs were counted and weighed using a 100 g Pesola® spring scale (± 0.5 g) once the clutch was complete. At 9 (± 1) days of age, the nestlings were uniquely identified with temporary colored leg bands, weighed using a 300 g Pesola® spring scale (± 1 g), their tarsal and wing chords measured (± 0.01 mm (digital caliper) and ± 1 mm (ruler), respectively), and right-wing web injected with phytohemagglutinin (PHA) to stimulate the T-cell based immune response. PHA response was checked 24 hours later (day 10).

Fifteen days after hatch the nestlings were weighed and measured. Two or three nestlings were randomly selected from each nest for euthanasia by cervical dislocation after anesthesia in a closed container containing an isoflurane-impregnated cotton swab. The liver of each nestling was removed within 5 minutes of euthanasia, trimmed of gallbladder and weighed; a thin-section was

preserved in formalin for histology and two ca. 500 mg portions were flash-frozen in cryovials, in liquid nitrogen, then stored at -80°C for EROD enzyme and oxidative stress biomarker analyses. The spleen and bursa of Fabricius were weighed, sections were preserved in formalin with the thyroids, gonads and liver sections for future histopathology studies.

3.3.3 Possible confounders: year, hatch date, weather

As with any field study, many factors may confound the interpretation of results.

One investigator performed all measurements in 2013 (pilot study), while a different investigator (MN) conducted all aspects of the study for 2014 and 2015. To control for the effects of operator differences in measurements and biochemical responses, individual nestling responses from 2013 were excluded from analysis for this paper. Reproductive responses (e.g., clutch size) are less sensitive to operator differences, and therefore those data from 2013 were included. Variation in environmental conditions and experimental methodology between years was accounted for by including year as a factor in all statistical analyses.

The timing of reproduction can significantly affect many reproductive and morphological variables. Birds ideally nest at an optimal time to ensure sufficient quality food for their brood, weather that promotes offspring survival, as well as enough time for the chicks to fully mature to maximize migration and overwinter survival. The date of hatch (Julian date, or ordinal calendar date) was included as a covariate in all models to account for this source of variance. The effect of weather on reproductive success and nestling growth was assessed using meteorological data archived by Environment Canada (2013–2015). Mean daily precipitation, total precipitation, mean minimum and maximum daily temperature, mean daily diurnal temperature range (max - min), and mean temperature (mean of maximum and minimum temperatures) were calculated for the week before and the week after the hatch date for each nest. Total precipitation included snow and rain.

3.3.4 Biological indicators

All animal procedures were approved by the University of Calgary Animal Care Committee, in accordance with Animal Use Protocols SHC11R-15 and AC15-0070.

3.3.4.1 Reproductive performance

Reproductive success, evaluated for 2013 to 2015 used the following endpoints: clutch size and mean egg mass, hatching success [(# hatched/clutch size) *100], and nest success [(# fledged/clutch size) *100].

3.3.4.2 Immune response

Effects of air pollutants on the cell-mediated immune response was evaluated using the phytohaemagglutinin (PHA) skin test, a method commonly employed in ecotoxicology as an indicator of immunocompetence (Grasman, 2002, Westneat *et al.*, 2004, Martin *et al.*, 2006). The method used is described in Smits *et al.* (1999). Phytohaemagglutinin (PHA-M, Sigma Aldrich, Inc., St Louis, MO, USA) diluted with sterile phosphate buffered saline (pH 7.4) to 1 mg/ml was injected intradermally into the right wing-web 9 days after hatch, using a dose of 30 μg per bird. The thickness of the wing web was measured in triplicate at the injection site using a spring-loaded caliper (The Dyer Company, Lancaster, PA, USA) immediately before injection, and 24 h (±1 h) later, with the difference representing the T-cell mediated inflammatory response. The same investigator measured all responses.

3.3.4.3 Body and organ morphometric indices

Nestling size and mass were measured on day 9 and 15 after hatch (± 1 day). Wing chord, a method of assessing the developmental maturity of nestlings, was measured using a modified ruler held against the carpal joint and measuring to the end of the longest primary feather with the wing gently flattened. Tarsal length, a proxy of skeletal size, was measured using digital caliper.

Nestling body condition was calculated using scaled mass index (SMI), a rigorous method of predicting an animal's body mass after standardizing its skeletal size (Peig and Green, 2009) which has been applied successfully in other wildlife toxicology studies (Lopez-Antia *et al.*, 2013, Rodríguez-Estival *et al.*, 2015). In this population, tarsal length was the best measure of skeletal size. The SMI for each nestling was calculated at 9 and 15 days of age as follows: $SMI_i = M_i [L_0/L_i]^{bSMA}$; where b_{SMA} = *slope/Pearson's r,* and SMI_i, M_i and L_i are the scaled mass index, body mass and tarsal length of the individual *i*, respectively. The reference length, L_0 was the mean tarsal length for all nestlings measured in 2013 to 2015 at days 9 and 15 separately, after removing

outliers. The scaling exponent (b_{SMA}) as described in Peig and Green (2009) and reference L_0 values used for calculating the SMI on day 9, were 1.879 and 29.32 mm, and on day 15, were 5.106 and 30.10 mm, respectively.

Liver, spleen and Bursa of Fabricius masses were normalized for body size using a modified version of the scaled mass index (Rodríguez-Estival *et al.*, 2015), scaled liver mass index (SLMI), scaled spleen mass index (SSMI), scaled bursal mass index (SBMI). Scaled organ mass indices were calculated using a modified version of the formula for SMI: $SoMI_i = OM_i [L_0/L_i]^{bSMA}$, where L_0 is the population mean for TL15 and L_i the tarsal length of the individual, as above, and $SoMI_i$ and OM_i are the scaled organ mass index and organ mass for individual *i*, respectively. The L_0 and b_{SMA} values used for liver, spleen and bursa were 30.10 mm, and 9.17, 17.99 and 12.38, respectively.

3.3.4.4 Hepatic EROD activity

The activity of cytochrome P4501A1 (CYP1A1) monooxygenase in the livers of nestling starlings was determined by measuring 7-ethoxyresorufin-*O*-deethylase (EROD) activity, following the standard protocol described by Nilsen *et al.* (1998), with modifications to buffers and incubation time described in the Supporting Information (p S4), and reported as pmol resorufin per mg protein per minute. The coefficient of variance (%CV) for duplicate samples and standards was 3.29 ± 2.44 and 3.01 ± 2.10 for 2014 and 2015, respectively, and the inter-assay %CV for standard curves was 14.58 ± 13.06 (mean ± SD).

3.3.4.5 Lipid peroxidation and GSH redox status in liver

Total glutathione (tGSH) and oxidized glutathione (oxGSH) were measured in 2014 using the methods and reagents described for liver samples in Rodríguez-Estival *et al.* (2015); thiobarbituric acid-reactive substances (TBARS) were measured using the same methods in both 2014 and 2015. Minor adjustments were made in 2015 to improve efficiency of the analysis of tGSH and oxGSH (see Supporting Information, p S4). Reduced glutathione (rGSH) and glutathione disulfide (GSSG) were calculated using the following formulae: rGSH=tGSH-oxGSH and GSSG=oxGSH/2 (Rodríguez-Estival *et al.*, 2015). We report TBARS, a measure of malonaldehyde reflecting membrane lipid peroxidation (Kelly *et al.*, 1998), as well as total glutathione (tGSH) and the ratio

of the reduced to oxidized forms of glutathione (rGSH:GSSG), since these latter two provide an indication of up-regulation of the antioxidant defense system (Koivula and Eeva, 2010). This combined, multi-pronged approach provides more complete insight into the actual oxidative stress from contaminant exposure, since any biomarker in isolation is inconclusive in such a redundant system. Thus, TBARS, which may overestimate lipid peroxidation levels, is complemented by the measurement of oxidized and reduced GSH.

The intra-assay coefficient of variation (%CV) for replicate samples for EROD and TBARS for 2014 and 2015 were all below 15%, for tGSH the %CV was 31% necessitating deletion of outlying values, and oxGSH in 2015 were below 5%. The oxidized GSH assay proved problematic in 2014, and therefore was excluded. Detailed information on quality control is available in Appendix B.

Chemicals for measuring EROD and oxidative stress biomarkers were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada) or VWR International (Edmonton, AB, Canada). Equipment used were spectrophotometric plate reader (SpectraMax Plus384 Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro software (version 6.0, Molecular Devices LLC, Sunnyvale, CA, USA) for protein and oxidative stress biomarkers, and benchtop ultracentrifuge (Beckmann and Coulter Inc., Chino, CA, USA), spectrofluorometer (Flexstation II, Molecular Devices) with SoftMax Pro 6.0 software for EROD.

3.3.5 Statistical analysis

To test the for effects of air pollutants on parent-bird reproduction, 'nest' was used as the experimental unit; for the effects of exposure on nestling starlings, individual records were analyzed using unique clutch ID to control for multilevel clustering (siblings within nests, nests within locations). Responses were tested for normal distribution (by year) (Shapiro-Wilk test for normality, $p<0.05$). If non-normally distributed, even after removing outliers, Spearman's correlation was used to detect associations among variables. Air quality variables were checked for collinearity using Spearman's correlation, and BTEX and hexane were chosen as the two proxy (indicator) pollutants best representing overall air contaminants. Difference in air quality among regions was checked using a One-Way ANOVA (with LSD), with BTEX, hexane and NO_2 included as dependent variables and region (n=5) as factor. Confounding factors were checked for

possible significance using Spearman's correlation (for continuous factors: HDJ and weather) or One-Way ANOVA (year). Significant confounders were included in further analyses using multivariate models.

Associations between biological responses and potential predictors (hatch date, weather and pollution concentration) were investigated using Spearman's correlation. The effect of exposure on nest-level reproductive data (egg mass, clutch size, hatch- and nest success, 2013 to 2015) was checked using Spearman's correlation, and analyzed using univariate generalized linear models, including hatch date, BTEX and hexane as covariates, year and location as factors.

The effect of exposure and relative importance of confounding predictors (year, hatch date and location) on individual nestlings' responses (2014 and 2015) was analyzed using generalized linear mixed models with generalized estimating equations (GEE) to account for the clustering of nestlings within each clutch, which was included as a random effect.

Two-tailed significance was set at $p<0.05$. Analyses were done using SPSS 24.0 statistical software (SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1 Air pollution: concentrations and distribution

Pollutants were measured at 3 sites in 2013, 23 sites in 2014, and 17 sites in 2015, and combined into five regions, each with multiple measurements from 2013 to 2015. The spatial and annual variability of air pollutant concentrations measured using passive samplers is represented in Figure 3-3.

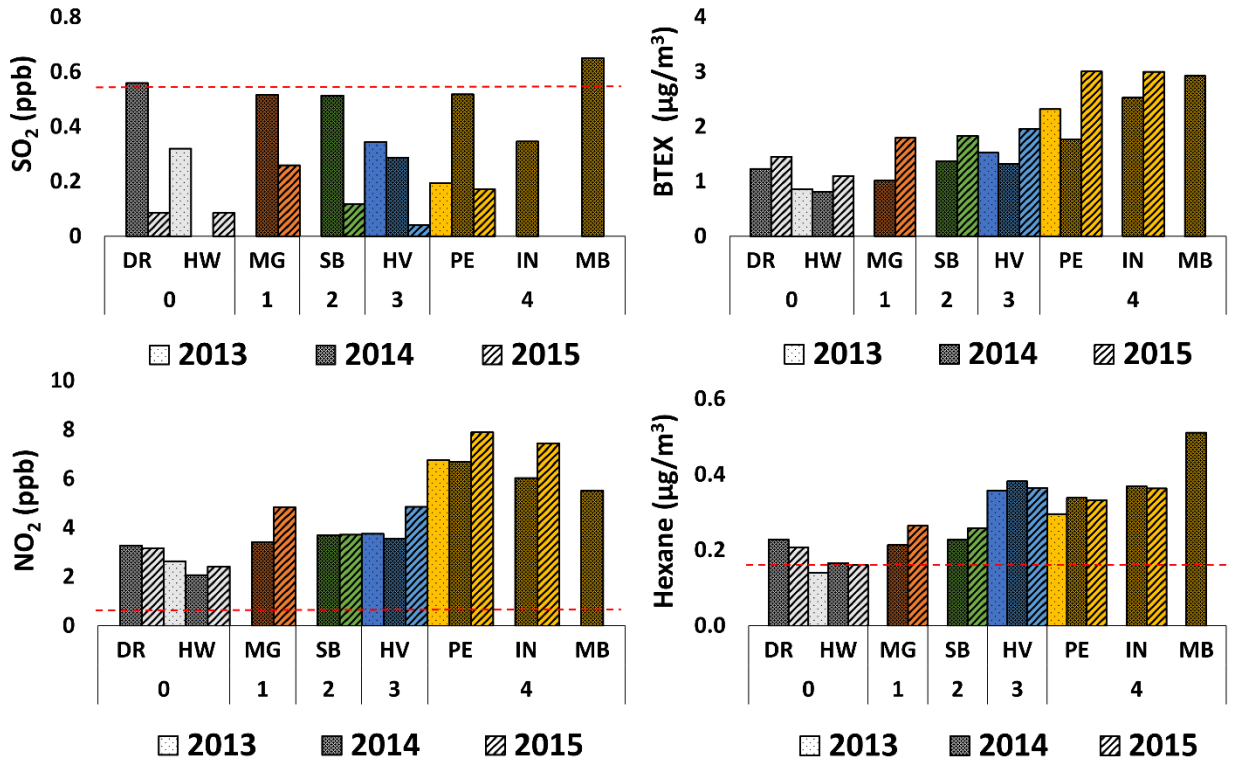

Figure 3-3. Concentrations of SO_2, NO_2, BTEX and hexane measured in 2013–2015, grouped by region (0 to 4, colors correspond with Figure 4) and location. Red dashed lines indicate the minimum limit of detection / quantification (LOD/LOQ, i.e., low confidence in the accuracy of SO_2 results); there is no red line for the integrated, multi-pollutant 'BTEX', since each of its constituent compounds has their own LOD/LOQ. The units used for NO_2 and SO_2 (ppb, parts per billion, also ppbv, or parts per billion volume) are the units reported in the U.S. EPA's National Ambient Air Quality Standards (NAAQS).

Since SO_2 concentrations were frequently below the manufacturer's reported detectable limits (Figure 3-3, Anon (2002)), it was not analyzed further. Similarly, some VOCs consistently below LOD/LOQ for multiple sites and years (Appendix B, Table B-3), were excluded from analyses.

Air quality indicators for 2013 to 2015 (passive samplers, n=22) had a high degree of correlation (all $p \leq 0.002$). Nitrogen dioxide (NO_2) was significantly correlated with hexane (Spearman's rho, $r_s=0.622$, $p=0.002$), benzene ($r_s=0.669$, $p=0.001$), heptane ($r_s=0.689$, $p<0.0001$), toluene ($r_s=0.946$, $p<0.0001$), ethylbenzene ($r_s=0.734$, $p<0.0001$), *m, p*-xylenes ($r_s=0.908$, $p<0.0001$), *o*-xylene ($r_s=0.808$, $p<0.0001$), and BTEX ($r_s=0.892$, $p<0.0001$). BTEX was significantly correlated with NO_2 ($r_s=0.892$, $p<0.0001$), hexane ($r_s=0.687$, $p<0.0001$), benzene ($r_s=0.845$, $p<0.0001$), heptane ($r_s=0.871$, $p<0.0001$), toluene ($r_s=0.971$, $p<0.0001$), ethylbenzene ($r_s=0.877$, $p<0.0001$), *m, p*-

xylenes (r_s=0.969, p<0.0001), and *o*-xylene (r_s=0.897, p<0.0001). Consequently, BTEX was selected as the proxy for exposure for all compounds (where r_s>0.8) except hexane.

Thus, BTEX, hexane and NO_2 concentrations are significantly different among regions in Calgary (F_4=12.084, 15.085 and 20.531 respectively, all p<0.0001) which justifies considering the different regions as distinct. The geographic location and NO_2, SO_2 and BTEX concentrations in each of the five regions of Calgary are shown in Figure 3-4.

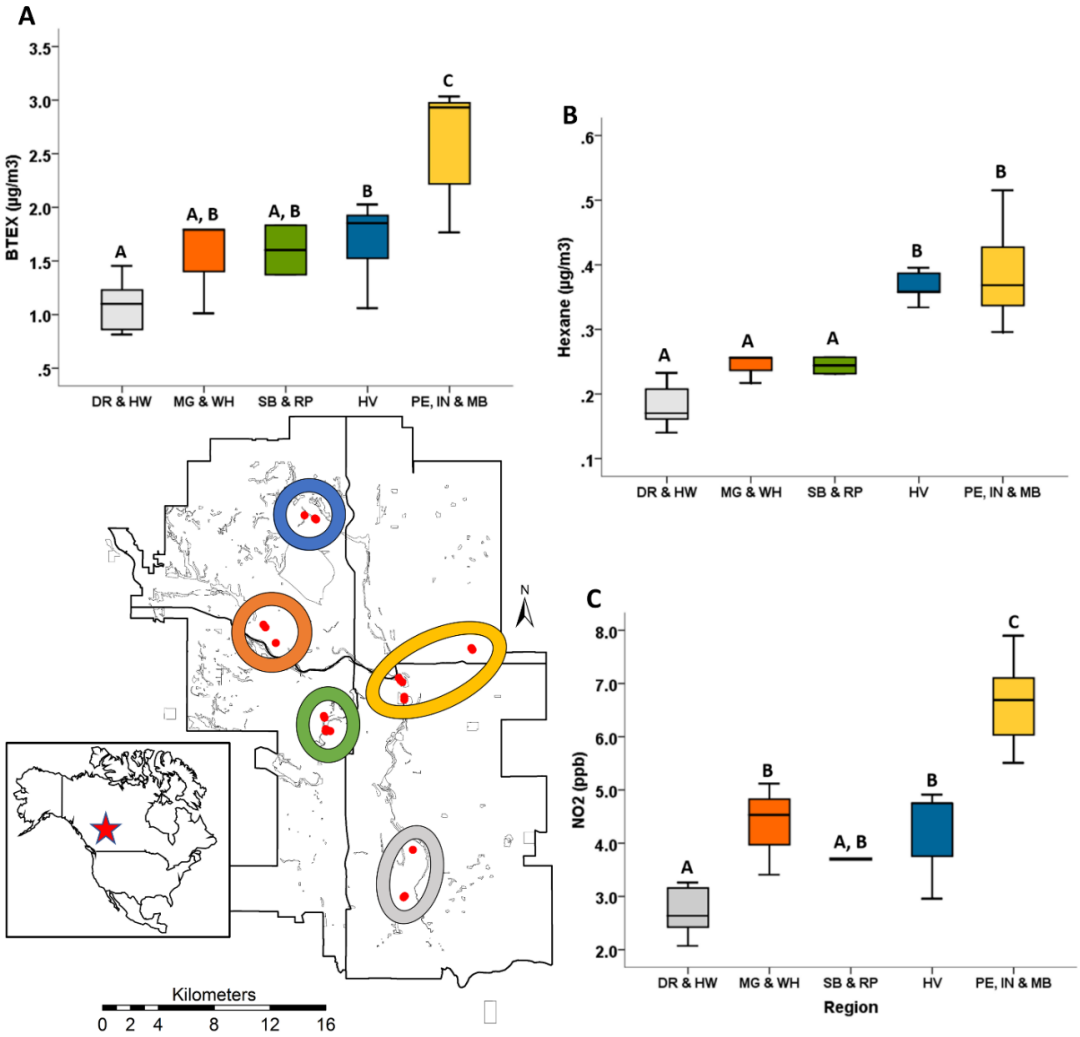

Figure 3-4. Box-and-whisker plots of contaminant concentrations measured in 2013–2015 (Inset graphs: A. BTEX, B. Hexane & C. NO_2) in five regions as indicated by the map of Calgary, AB, Canada (lower left) [1]. Significant differences in concentration between regions are marked with uppercase letters within each graph.

[1] Maps created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. Source: City of Calgary. 2017 City of Calgary quadrants and parks .shp files. Created by University of Calgary Library Spatial and Numeric Data Services (SANDS), using ArcGIS 10.5 (June 06, 2017).

To assess the biological responses to exposure to urban air pollution, we will focus only on the effects of BTEX (and, by proxy, NO_2) and hexane on the biological responses.

3.4.2 Reproductive and nestling responses

Forty-seven clutches in nine locations are included in this analysis; 4 in 2013, 24 in 2014 and 19 in 2015. All but four clutches fledged at least one chick. Nestling mortality was rare, with only five clutches having less than 100% survival; four clutches lost one nestling each, and one, four-nestling nest had 100% mortality.

The mean and standard deviation (SD) for the reproductive and nestling responses are listed in Table 3-2, organized by location. The large SDs observed in EROD and the ratio of rGSH to GSSG prompted further investigation and the separation of results from the two years during further analyses.

Table 3-2. Distribution (mean ± SD) of the results for reproductive (2013–2015) and individual (2014–2015) responses.

	Region	0		1		2		3	4		
	Location	HW	DR	MG	WH	RP	SB	HV	PE	IN	MB
Reproductive	Clutch size	4.6	5	5	5.5	5	5.2	4.6	4.7	4	6
	Egg mass [a]	6.75 ± 0.71	6.54 ± 0.80	5.55 ± 0.57	7.21 ± 0.59	7.08 ± 0.91	6.39 ± 0.52	6.75 ± 0.71	6.78 ± 0.61	5.97 ± 0.30	6.06 ± 0.0
	Hatch success	25 ± 43	78 ± 15	81 ± 29	100 ± 0	86 ± 19	65 ± 39	91 ± 16	91 ± 10	72 ± 16	83 ± 24
	Nest success [b,]	25 ± 43	78 ± 15	78 ± 30	100 ± 0	86 ± 19	65 ± 39	74 ± 36	91 ± 10	68 ± 10	75 ± 12
Nestling Day 9	Body mass [a]	56.8 ± 15.9	62.7 ± 4.9	58.6 ± 7.7	64.3 ± 3.3	61.3 ± 6.3	56.6 ± 7.2	59.5 ± 8.1	60.6 ± 7.9	61.8 ± 6.2	65.0 ± 6.6
	Wing chord [c]	45.7 ± 5.0	48.1 ± 6.8	46.4 ± 6.5	48.5 ± 5.3	51.7 ± 7.0	43.0 ± 6.0	44.8 ± 5.4	43.1 ± 6.5	49.0 ± 7.4	53.1 ± 4.5
	Tarsal length [c]	28.7 ± 1.3	29.1 ± 1.0	28.5 ± 1.1	29.4 ± 0.7	29.0 ± 0.8	28.7 ± 1.2	28.6 ± 1.3	28.6 ± 1.2	29.1 ± 0.8	29.2 ± 0.9
	SMI	60.2 ± 8.0	64.3 ± 5.0	63.5 ± 3.8	64.1 ± 3.4	62.7 ± 5.1	60.0 ± 3.52	64.3 ± 7.3	65.8 ± 5.4	63.4 ± 6.4	65.6 ± 3.7
	PHA response	0.25 ± 0.19	0.61 ± 0.23	0.75 ± 0.17	0.57 ± 0.19	0.63 ± 0.27	0.56 ± 0.16	0.49 ± 0.18	0.72 ± 0.32	0.66 ± 0.30	0.30 ± 0.17
Nestling Day 15	Body mass [a]	73.7 ± 1.1	67.0 ± 7.8	71.1 ± 8.1	76.8 ± 8.5	68.2 ± 3.4	68.0 ± 7.6	71.3 ± 8.7	67.6 ± 11.1	68.5 ± 8.3	73.9 ± 7.3
	Wing chord [c]		83.4 ± 2.9	87.8 ± 5.6	85.5 ± 2.3	85.4 ± 3.0	79.6 ± 3.1	83.7 ± 4.3	82.6 ± 3.8	86.1 ± 7.0	
	Tarsal length [c]	30.3 ± 0.5	30.2 ± 0.5	30.0 ± 0.8	30.9 ± 1.2	29.8 ± 0.8	30.1 ± 0.6	30.1 ± 0.8	30.2 ± 0.8	30.1 ± 0.5	29.7 ± 0.7
	SMI	64.7 ± 5.0	62.3 ± 5.1	69.6 ± 6.5	65.3 ± 9.4	69.2 ± 10.3	64.0 ± 9.5	68.8 ± 7.0	62.1 ± 9.7	64.9 ± 7.9	73.4 ± 7.4
	ΔSMI	4.6 ± 12.8	-2.3 ± 6.5	5.3 ± 6.2	1.7 ± 8.8	6.6 ± 13.4	3.2 ± 9.8	5.8 ± 11.0	-4.2 ± 8.4	2.1 ± 7.9	7.2 ± 5.5
Lethal biomarkers	SSMI	0.05 ± 0.02	0.10 ± 0.03	0.12 ± 0.06	0.10 ± 0.02	0.13 ± 0.08	0.09 ± 0.04	0.10 ± 0.05	0.10 ± 0.04	0.10 ± 0.03	0.12 ± 0.07
	SBMI	0.17 ± 0.10	0.14 ± 0.03	0.16 ± 0.04	0.16 ± 0.03	0.14 ± 0.06	0.13 ± 0.06	0.16 ± 0.04	0.12 ± 0.05	0.15 ± 0.05	0.16 ± 0.06
	SLMI	3.21 ± 1.11	2.70 ± 0.29	3.53 ± 0.76	2.81 ± 0.35	3.48 ± 1.30	3.05 ± 0.92	3.17 ± 0.96	2.51 ± 0.63	2.94 ± 0.53	4.25 ± 1.22
	EROD [2]	107.2 ± 38.2	173.1 ± 124.6	269.4 ± 213.9	98.6 ± 78.5	224.2 ± 171.2	178.9 ± 98.8	120.6 ± 108.2	172.6 ± 113.9	277.9 ± 236.4	89.9 ± 19.6
	TBARS	46.73 ± 3.78	58.24 ± 12.3	51.19 ± 13.60	57.69 ± 8.97	41.84 ± 14.95	47.75 ± 7.64	48.40 ± 12.76	51.57 ± 10.39	43.20 ± 11.81	55.76 ± 6.26
	tGSH	6.03 ± 1.20	4.72 ± 1.41	6.62 ± 1.22	5.26 ± 0.99	4.42 ± 1.62	4.87 ± 1.89	4.64 ± 1.50	4.63 ± 1.76	5.67 ± 2.12	5.77 ± 1.71
	rGSH:GSSG [2]	11.81 ± 3.77	21.79 ± 15.20	29.53 ± 20.44	24.56 ± 21.78	32.48 ± 25.56	16.81 ± 12.08	22.57 ± 13.95	22.68 ± 17.31	33.27 ± 19.08	14.32 ± 3.15

[1] Nest success includes both the proportion of eggs hatched (hatch success) and the survival of fledglings until able to leave the nest, which is why it sometimes has the same value as hatch success (i.e., no nestling mortality). [2] These results include all data for both years together, thus the large standard deviations. Later analyses are segregated by year to correct for this. Measured in [a] grams, [b] percent, [c] millimeters.

3.4.3 Confounding factors

Mean precipitation (week before and after hatch) was significantly different among years ($F_{2,44}$=51.37, p<0.0001, and $F_{2,44}$=6.52, p=0.003, respectively), with 2013 having more precipitation than 2014 and 2015. Maximum temperatures (before and after hatch; $F_{2,44}$=5.25 and 5.49, p=0.009 and 0.007, respectively), diurnal temperature range (after hatch; $F_{2,44}$=15.64, p<0.0001) and the average temperature (before hatch; $F_{2,44}$=3.98, p=0.026) were also different among years. Daytime temperatures were significantly cooler in 2013 than 2014 or 2015. Of the biological variables, year was significant for scaled mass index on day 15 (and consequently the change in SMI from day 9 to 15; $F_{1,127}$=12.49, p=0.0091 and $F_{1,123}$=9.93, p=0.003, respectively), the size-adjusted mass of the bursa of Fabricius (SBMI; $F_{1,96}$=4.53, p=0.040), and the following biomarkers (EROD, TBARS, tGSH and rGSH:GSSG; $F_{1,101}$=77.10, 7.50, 11.64 and 80.75, p<0.0001, = 0.009, 0.002 and <0.0001, respectively). Including year as a factor in subsequent analyses accounts for the effects of precipitation and temperature, and controls for differences in laboratory conditions for EROD, TBARS, tGSH and rGSH:GSSG.

For 2013–2015, hatch date (HDJ) is significantly correlated with minimum temperatures for the week before and after hatch (r_s=0.853 and 0.838, respectively, p<0.0001), maximum temperatures for the week before and after hatch (r_s=0.677 and 0.635, respectively, p<0.0001), diurnal temperature range for the week before hatch (r_s=0.427, p=0.003), and average temperatures for the week before and after hatch (r_s=0.789 and 0.778, respectively, p<0.0001). Thus, as the breeding season progresses (increasing HDJ), we expect warmer temperatures (but minimal change in the diurnal temperature variation). Inclusion of HDJ in analyses serves as a proxy for the effects of temperature on nestling outcomes. The biological responses correlated with hatch date include: day 9 and 15 wing chord (r_s=0.216, p=0.006, n=160 and r_s=0.469, p<0.0001, n=61, respectively), day 15 tarsal length (r_s=-0.240, p=0.006, n=128), liver somatic index (r_s=-0.200, p=0.041, n=105), and liver EROD (r_s=-0.317, p=0.001, n=104).

Weather was an important determinant of much of the biological variation, with temperature being the only significant predictor of nestling mortality (e.g. Gentes *et al.* (2006)). Detail on correlations

among temperature, precipitation and biological responses may be found in Appendix B. For this study, HDJ and year, reflecting weather, are included as covariates in analyses.

3.4.4 Effect of predictors: confounders and exposure to air pollution

Table 3-3 summarizes the results of this study, emphasizing the relative importance of possible predictors, including confounding factors, on the responses being investigated. Year was not a significant determinant of reproductive success, as measured by clutch size, egg mass, hatch- and nest-success, and therefore was excluded from further analyses. The relative contribution of factors on nest-level reproductive responses found that location was a significant predictor for all the reproductive measures other than clutch size ($p<0.05$, Table 3-3), with Inglewood and Montgomery having lighter eggs (B=-2.071, $p=0.028$ and B=-1.659, $p=0.006$, respectively), and Hull's Wood a lower hatch- and overall nest success (B=-82.091, $p=0.003$, and B=-85.128, $p=0.007$, respectively), compared with the other locations. Contaminant exposure was not a significant predictor of egg mass or overall reproductive success; however, clutch size decreased with increasing BTEX (Spearman's correlation: $r_s=-0.499$, $p=0.001$, n=44, and generalized linear models: Table B-6).

Location was the most important predictor of morphometric measurements, oxidative stress and detoxification responses, with hatch date and year also contributing to variation (Table 3-3, and Table B-7). The concentration of BTEX was a significant predictor of day 9 mass and SMI, day 15 tarsal length, the change in SMI from day 9 to 15, and total glutathione (tGSH), while hexane was only significant for tGSH. The interaction between BTEX and hexane was significant for all the same responses as BTEX alone, although incurring the opposite effect, suggesting antagonistic modes of action.

Table 3-3. Relative contribution of the different predictors included in each model on the responses measured in this study.

Responses			n_s	$n_{w/s}$	Predictors: Year	Location	Hatch date	BTEX	Hexane	BTEX-hexane interaction
Reproductive†		Clutch size	44					-		
		Egg mass	43			X				
		Hatch success	44			X				
		Nest success	44			X				
Nestling‡	Day 9	Body mass	37	148		X		+		-
		Wing chord	37	149		X				
		Tarsal length	37	145		X	-			
		SMI	37	144	X	X		+		-
		PHA	37	134		X				
	Day 15	Body mass	37	122		X	-			
		Wing chord [a]	14	61		X				
		Tarsal length	37	117	X	X	-	+		-
		SMI	37	118	X	X				
		ΔSMI	37	114	X	X		-		+
	Lethal biomarkers	SSMI	36	90		X				
		SBMI	36	90		X				
		SLMI	36	90	X	X	+			
		EROD (2014) [b]	20	72		X	-	+		-
		EROD (2015) [b]	13	32		X				
		TBARS	36	95	X	X	-			
		tGSH	36	95	X	X		-	-	+
		rGSH:GSSG [c]	13	31		X				

Sample size (ns) refers to the number of subjects used in each model (unique clutch ID, which includes nest box #, year and brood #), while nw/s refers to the within-subject sample size (total number of individual nestlings), with variation due to missing air pollution data or selective sampling for euthanasia. Significant predictors ($p<0.05$) are indicated with an X (categorical predictors), or + or – (continuous predictors, indicating direction of effect). Grey boxes indicate predictor(s) not included in the model for that response. [a] Only data from 2015. [b] Difference between years obscured underlying, subtle effects of location, hatch date and pollutants on EROD. [c] The oxidized GSH assay in 2014 proved problematic and was excluded. † Univariate generalized linear models: 1) Intercept + hatch date + BTEX + hexane + year, 2) Intercept + hatch date + BTEX + hexane + location. ‡ Generalized mixed effects models with generalized estimating equations; subject = clutch ID, within subject = individual nestlings, factors = year and location, covariates = hatch date and pollutant concentrations, with BTEX * hexane interaction effect included. For

models analyzing years separately, subject = box # instead of clutch ID; relative effects of air pollution and location had to be evaluated separately.

3.5 Discussion

Research into air pollution toxicology has classically focused on high concentration, single-pollutant exposure of laboratory species, describing physiological effects and dose-response relationships. While these studies support health-related legislation such as the U.S. EPA's Integrated Science Assessments for Oxides of Nitrogen (2016), Sulfur Oxides (2008) and Particulate Matter (2009), and the U.S. National Ambient Air Quality Standards (NAAQS), they do not advance our understanding of the health effects of commonly occurring pollutant mixtures. Advances in epidemiological modelling have facilitated studies into health risks associated with exposure to air pollution; while these studies have real-world relevance for policy-makers, the reliability of computational models depends on highly detailed, good quality population data that can be stored and accessed from a secure central health database. Their relevance relies on detailed air quality measurements collected over a prolonged timeframe. Many developing countries lack capacity and resources to operate such a framework, and developed countries struggle to obtain such data for small towns and remote communities.

Monitoring the health of wildlife populations could provide early-warning of potential health effects from air pollution. The short lifespan, high metabolic rate and exceptionally efficient respiratory system make it likely that health effects will manifest in birds before being observed in humans, and could trigger vigilance by health services. The biomarkers measured in this study were chosen because they are well-conserved across vertebrate species. Ideally, biomarkers used for health surveillance should be practical, relatively simple under field conditions, cost effective, and repeatable by different investigators.

Urban SO_2 concentration is usually related to heavy transport using diesel fuels, or from shipping in harbor cities. Calgary's low SO_2 concentrations may reflect the limited use of diesel fuel due to the cold climate. Whereas NO_2 has been used as the single, proxy air pollutant in numerous epidemiological studies, this study found the combination 'BTEX' to be highly correlated with most measured VOCs and NO_2, making it the best choice as proxy of exposure, plus hexane. With the co-emission of many of these contaminants from vehicles, vehicle traffic is likely the greatest

source of variation of the pollutants measured in Calgary. The unexpectedly high concentration of hexane alone in one location suggests a source unrelated to vehicles, possibly the storage tanks of a nearby gas station (< 600 m from nest sites), or less likely, from the municipal landfill (ca. 6 km away) or unknown industries using it as a solvent (Agency for Toxic Substances and Disease Registry (ATSDR), 2004).

Reproductive endpoints proved relatively insensitive, with only clutch size showing any relationship with air pollutants. Reproduction, a key determinant of population survival, would be robust, particularly in species as successful as European starlings. Non-invasive, "ideal" biomarkers of growth and development, nestling mass, condition and skeletal size, were sensitive to exposure to BTEX and BTEX-hexane interaction, confirming their value in environmental monitoring. The invasive, generally sensitive biomarkers, liver detoxification effort (EROD) and total GSH, were significantly associated with exposure. However, inter-assay variation limited multi-year comparisons, sample size, and sensitivity. Hexane exposure reduced the nestlings' total GSH. The most consistent predictor of significant responses to contaminant exposure was geographic location, confirming importance of location which would account for confounders like food availability, disturbance, noise and light pollution, etc. The effect of hatch date and year (and thus, weather) was smaller than expected.

The apparent negative effect of BTEX on clutch size may occur through endocrine disruption, follicular atresia and absorption, which could reduce clutch size (Johnson, 2015). BTEX compounds have been associated with reproductive dysfunction (low birth weights (Aguilera *et al.*, 2009), increased risk of spontaneous abortion (Xu *et al.*, 1998)). A review by Bolden *et al.* (2015), reports a myriad of endocrine effects, supporting the biological plausibility that exposure of the females early in the breeding season may compromise their reproductive productivity. An alternative explanation is that the highest quality pairs select nesting areas with lower BTEX and related impacts, displacing first-year or suboptimal breeding females.

Growth indicators and total glutathione were the most sensitive to BTEX, while accounting for other major confounding influences. Incongruously, birds in parks with higher BTEX concentrations were heavier, with higher body condition than their cleaner counterparts, despite elevated detoxification activity and lower total antioxidant reserves. In this study, nestlings

maintained their capacity to cope with the oxidant burden, evident by stable rGSH:GSSG ratios. Co-pollution with hexane had opposite effects, with co-exposed birds being smaller, with poorer body condition, lower EROD and more tGSH than their counterparts, suggesting some physiological interaction of pollutants in the nestlings, as has been demonstrated between benzene and toluene in people (Inoue *et al.*, 1988), and American kestrels (Olsgard *et al.*, 2009). The nature of any such interaction between the BTEX compounds and hexane is not possible to tease out without further, experimental investigation.

Air contaminants including benzene, NO_2 and SO_2, are thought to increase oxidative stress via various mechanisms (Livingstone, 2001). Here, tGSH was one of the few biomarkers sensitive to both BTEX and hexane, with lower antioxidant reserves in areas with higher pollution. This suggests chronic exposure to oxidizing contaminants, agreeing with the downward trend in antioxidant capacity observed in House sparrows by Herrera-Dueñas *et al.* (2014). The lack of difference in rGSH:GSSG ratio in this study suggests that, although contaminant exposure reduced their total GSH, the birds maintained their reserve capacity to deal with oxidative stress. Our findings are different from others in which contaminant exposure has exhausted antioxidant resources in wildlife species (Koivula and Eeva, 2010, Rodríguez-Estival *et al.*, 2015). Although the laboratory technique to determine rGSH:GSSG is challenging and the accuracy of results has been questioned (Giustarini *et al.*, 2016), we argue that precision in analyzing samples within one study, produces meaningful comparative results.

Field studies of air contaminants are limited, however, a recent investigation in Sweden on great tits (*Parus major*), found significant negative impacts on oxidative stress, plumage color and body condition in urban birds when compared to their rural counterparts (Isaksson *et al.*, 2005). In adult American kestrels (*Falco sparverius*) exposed to a mixture of benzene, toluene, NO_2 and SO_2 (concentrations 1,000–10,000 times higher than this study), exposed birds had significantly lower thyroid hormones and altered thyroid histology when compared to controls (Fernie *et al.*, 2016). Cruz-Martinez *et al.* (2015a) exposed Japanese quail (*Coturnix coturnix japonica*) and American kestrels to the same mixture of contaminants, found species and concentration differences in responses, with only EROD and plasma corticosterone increasing in response to exposure.

Traffic noise for passerines has been considered as a stressor, but other, non-noise related effects of traffic proximity such as pollution, may pose the actual stress (Crino *et al.*, 2013). Traffic noise was not considered here, although none of the nests were near large roads. Extreme weather events overwhelm subtle effects of chronic toxicant exposure, as was evident in this and other studies (Gentes *et al.*, 2006). Investigators should include temperature and precipitation data in field-based toxicology studies.

None of the contaminants measured in this study approached Alberta, WHO or US-EPA ambient air quality guidelines for human health (Table 1-1). Many of the guideline thresholds are based on single-pollutant toxicology studies using rodent models. Safety factors are built in for human health, however, the relevance to chronic exposure to mixtures of contaminants on non-laboratory species such as wild birds have not been established.

Because of the generally low pollution in Calgary, differences in reproductive success and development of the nestlings are likely substantially affected by weather (rainfall and temperature), and food quality and quantity, local (approved) pesticide application, human disturbance, etc. Habitats were matched as well as possible for the nest boxes, possible confounding factors were incorporated in the analyses as completely as possible, and a thorough suite of non-lethal and post-mortem studies on the birds, make this a comprehensive study into the effects of exposure to urban, traffic-related air contaminants.

Future work should include a captive study controlling for the many major uncontrollable confounding influences, such as variation in weather, food, water, public interference and pesticide application. A captive study could further validate the biomarkers used here, for exposure to comparable pollutant mixtures and at environmentally-relevant concentrations, preferably using wild avian species appropriate for future environmental monitoring. Additionally, the mechanisms by which BTEX and hexane affect the significant responses, and the interactions between the pollutants should be explored in greater detail.

As air pollution becomes increasingly recognized as major contributor to death and disability globally, organizations focusing on large-scale, big-data research such as that performed by the WHO, US EPA and the Health Effects Institute will provide up-to-date evidence to support

environmental policy. However, for these reports to remain current it is crucial that scientific methods include a multi- or interdisciplinary approach, considering the effects of mixtures, chronic exposures, and the effects on non-laboratory species, thus improving human, animal and environmental health.

Supporting Information (Appendix B). Materials & Methods: additional detail (possible confounders, modifications to lab techniques and lab test quality control); tables (Table B-1 to B-7) and figures (Figure B-1 and B-2) of more detailed results and to support Supporting Information M&Ms. This material is available free of charge via the Internet at http://pubs.acs.org.

Chapter Four: **Biomarker sensitivity to vehicle exhaust in experimentally exposed European starlings**

4.1 Abstract

The effects of vehicle-related emissions on health has been a long-standing question in human health sciences; however, the toxicology of chronic exposure to environmentally relevant concentrations of these complex mixtures has not been characterized in wild birds. Adult European starlings (*Sturnus vulgaris*) were exposed to vehicle emissions, with combined benzene, toluene, ethylbenzene and xylenes (BTEX) concentrations totaling 13.3 $\mu g/m^3$ over 20 days of exposure for 5h per day. Exposed birds had significantly lower cell-mediated immunity (measured using phytohaemagglutinin skin test, $p<0.0001$), thyroxine (T4, $p=0.042$) and glutathione (GSH, $p=0.034$) concentrations than control birds. There was no difference in body condition, antibody response to vaccination, triiodothyronine (T3), hepatic biotransformation (7-ethoxyresorufin-*O*-deethylase activity) or oxidative stress (thiobarbituric acid-reactive substances and ratios of reduced to oxidized GSH) or organ masses between exposed and control birds. This study supports findings of previous studies examining wild birds exposed to these air contaminants, and raises concern that environmentally-relevant concentrations of common urban volatile pollutants may have measurable effects on health.

4.2 Introduction

Air pollution is an acknowledged physical determinant of human health (National Research Council (US) and Institute of Medicine (US), 2013, Office of Disease Prevention and Health Promotion (ODPHP), 2014), with exposure to ambient (outdoor) air pollution resulting in diverse disease conditions and globally causing 3.7 million premature deaths in 2012 (WHO, 2016). Traffic is a major source of air pollutants, many of which are implicated in negative effects on health (Kampa and Castanas, 2008, Brauer *et al.*, 2013). Among the pollutants associated with traffic, nitrogen dioxide (NO_2), sulfur dioxide (SO_2), particulate matter (PM, particularly $PM_{2.5}$), polycyclic aromatic hydrocarbons (PAHs), and the volatile organic compounds (VOCs) benzene, toluene, ethylbenzene and xylenes have been linked with adverse health effects in numerous

epidemiological and experimental studies (Lin *et al.*, 2004, Jerrett *et al.*, 2009, Patel *et al.*, 2010, Bolden *et al.*, 2015).

The effects of air pollution on wildlife, particularly after industrial accidents, has been widely noted, serving to enhance public awareness of some the ecological consequences of anthropogenic activity (Carson, 1962, Newman, 1979, Pandey *et al.*, 1986). The avian respiratory system has specialized adaptations optimising gas exchange compared with mammalian lungs, both at rest and during exercise, making birds more sensitive to many airborne compounds and, therefore, early sentinels of inhaled toxicants (Brown *et al.*, 1997). Many species of wild birds share our urban outdoor environment, potentially providing insight into the effects of urban contaminants on the health of other urban residents (Drasch *et al.*, 1987, Sicolo *et al.*, 2010, Bókony *et al.*, 2012, Herrera-Dueñas *et al.*, 2014). Field studies provide insight into some of the possible effects of air pollution on wild birds in a natural context (Cruz-Martinez *et al.*, 2015a), but the many confounding factors inherent to field research make it difficult to detect subtle changes in health (North *et al.*, 2017).

The effect of vehicle exhaust on animals under controlled, laboratory conditions has been investigated in typical laboratory species (i.e., rats, mice and guinea pigs, Murphy *et al.* (1963)), using whole vehicle exhaust diluted to ambient or elevated concentrations, and in birds using mixtures of two or more chemicals to simulate exposure (Olsgard *et al.*, 2009, Cruz-Martinez *et al.*, 2015b). While these animal studies have contributed to understanding the toxic mechanisms underlying the effects of exposure to traffic-related air pollutants, either the study species, pollutant profile or pollutant concentrations were not relevant to natural exposure scenarios, and provide limited insight into the consequences of chronic, ambient exposure on urban wild birds.

In urban areas, polycyclic aromatic hydrocarbons (PAHs), a group of compounds with known health effects, are predominately emitted in vehicle exhaust (Shen *et al.*, 2011). However, PAH emissions have decreased substantially in the last decades, particularly in developed countries with stringent emissions regulations (Shen *et al.*, 2011), the exhaust of gasoline vehicles contains considerably lower PAH concentrations than that of diesel vehicles, and that of idling vehicles contains lower concentrations than accelerating vehicles (Zielinska *et al.*, 2004, Wei *et al.*, 2015). For these reasons, and due to financial constraints, PAHs were not measured in this study.

We used a controlled experimental trial to identify causal linkages between environmentally relevant levels of vehicle exhaust and effects on avian health, expanding on work that has been performed previously in the field (North *et al.*, 2017). Biological outcomes (i.e., body condition, T- and B-cell immune responses, hepatic detoxification and oxidative stress, liver, spleen and testes mass, thyroid hormones and histology) were chosen because they are meaningful indicators of avian health. These responses have proven sensitive in various avian species using both natural air pollution and experimental mixtures of air contaminants common to urban environments (Olsgard *et al.*, 2008, Herrera-Dueñas *et al.*, 2014, Cruz-Martinez *et al.*, 2015b, Fernie *et al.*, 2016, North *et al.*, 2017).

The objective of this study is to test which biological responses are most sensitive to exposure to vehicle exhaust, to serve as biomarkers in future research into the effects of air pollution on resident wild birds.

4.3 Materials and Methods

4.3.1 Experimental setup

Design and implementation of this experiment was overseen by University of Calgary Occupational Health & Safety; all animal-related housing, handling and procedures per the University of Calgary Animal Use Protocol AC15-0176.

4.3.1.1 Enclosures, housing and enrichment

Two enclosures, 2 m x 6 m x 2 m high, were constructed adjacent to one another inside a weather-proof Quonset. An aspect ratio (length/height) of 3 and approximately 0.8 m^3 per bird, exceeded recommended requirements for starlings (Asher and Bateson, 2008, Bateson and Feenders, 2010). Enrichment included ropes, chains and branches of varying thickness for perching, natural mulch substrate to promote natural probing and gaping feeding behavior, water baths for preening and refugia. Each enclosure had two 0.8 x 1.15 m feed trays lined with a non-slip, artificial grass surface, providing at least 0.06 m^2 of feeding space per bird. Two poultry bell-waterers and a large, shallow dish of water were included for each enclosure, and cleaned daily. Fresh *Eragrostis* hay, apples and suet were provided occasionally for stimulation and energy. Natural lighting,

photoperiod and environmental temperatures (0–32 ℃) were not controlled. Enclosures had three 1x1 m windows that were opened to allow fresh airflow when exposure was not taking place.

These enclosure characteristics and group-housing of starlings, a naturally gregarious species, ensured that stress of captivity was reduced as much as possible, to minimize social stress as a confounder for study results.

4.3.1.2 Animals

Adult, wild European starlings (*Sturnus vulgaris*) were trapped with "M-traps" baited with apples in the Okanagan Valley, British Columbia, as part of an ongoing population control program. The population of starlings in this region are largely non-migratory, and the birds trapped for this study were all from a limited geographic area and habitat type (vineyards and orchards), therefore it is reasonable to assume similar background exposures. Birds were allocated randomly to groups after blocking by sex, identified with unique leg bands, weighed, and measured before exposure, during the adaptation period. After the one-month adaptation period, they were weighed again and 0–90 µL blood drawn from the jugular vein using heparinized 27G needles on 3 mL syringes. Birds were in captivity for a total of 2 ½ months before euthanasia by cervical dislocation after isoflurane-induced anesthesia.

The color of the base of the bill (pink for females and blue for males) and iris color were used to sex the starlings, as per Kessel (1951), with only one bird misclassified (verified during necropsy). A combination of unmedicated duck starter (20% vegetable protein) and pelleted horse feed was provided every morning (*ad lib*, approximately 37 g and 17 g per bird, respectively) before handling procedures or exposures. The starlings showed normal behavior (singing, eating, and bathing) when the investigators were not present in the enclosures, adapting well to confinement and the new food source. All birds gained condition (as calculated using the scaled mass index, see 4.3.2.1) during adaptation and maintained condition for the duration of the experiment. Seven of the 61 birds escaped during the adaptation period, however, no birds were lost during experimental exposure.

Observers were not blinded during sampling or measurement of the birds in this experiment, but were blinded for all laboratory and histopathology analyses.

4.3.1.3 Exposure

Control and exposed enclosures were adjacent to one another, 2 m apart, controlling for the effect of noise and investigator presence (Figure 4-1). Enclosures were sealed using heavy grade polyethylene sheeting, and unidirectional fans maintained positive pressure with clean ambient air in the control cage, and negative pressure in the exposed enclosure, to minimize cross-contamination of the groups. A light-duty 2010 gasoline pickup truck (U.S. EPA, 2008a) was used as the source of emissions, with its exhaust directed into the exposed enclosure for 5 hours per day, 6 days per week for a total of 20 days of exposure.

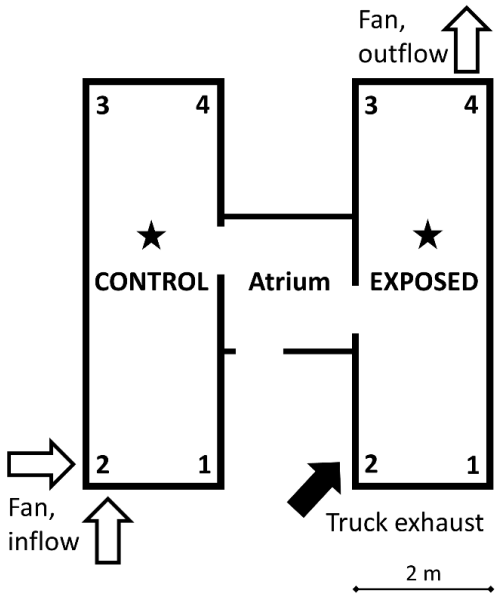

Figure 4-1. Layout of experimental and control enclosures. Passive samplers measuring NO_2, SO_2, and VOCs were suspended at the numbered corners, active monitors measuring $PM_{2.5}$ and VOCs were stationed at the stars.

Volatile organic compounds were measured using passive samplers (3M^{TM} Organic Vapor Monitor 3500, USA) attached in each of the four corners of the control and exposed enclosures for the duration of the experiment. Two field blanks were used to confirm correct handling and storage of the samplers. Nitrogen dioxide, sulfur dioxide and particulate matter (2.5 µm) were measured and found to be not different between the enclosures.

Over the month of exposure, benzene, toluene, ethylbenzene and xylenes concentrations (B+T+E+X, or BTEX) were 2.18 µg/m^3 in the control enclosure, and 13.3 µg/m^3 in the exposed

enclosure, with peak concentrations reaching approximately 62 µg/m^3 during the period of active exposure each day. The BTEX concentrations in this experiment were comparable to those measured in Barcelona, Spain (Aguilera *et al.*, 2009), and in Beijing under relatively clean conditions, during the 2008 Olympic Games (Liu *et al.*, 2009). Experimental concentrations were approximately 10 times those measured in Calgary, Alberta, during 2013–2015 (North *et al.*, 2017) (Table 4-1).

Table 4-1. BTEX concentrations (mean ± SEM, µg/m^3) in control and exposed enclosures, compared to mean concentrations measured in cities in Canada, Spain and China

		B	T	E	X	BTEX
Exposed		2.75	4.78	0.72	5.35	13.60 ± 2.94
Control		0.26	1.06	0.10	0.72	2.14 ± 0.13
Comparative levels:						
Canada	Calgary, AB (summers of 2013–2015) [a]	0.25	0.80	0.13	0.69	1.86
	Windsor, ON (autumns of 2004–2006) [b]	0.79	2.87	0.49	2.07	6.12
Spain	Sabadell, Barcelona (2004–2006) [c]	n/c	n/c	n/c	n/c	14.7
China	Beijing (during 2008 Olympic games) [d]	2.37	3.97	1.92	5.41	13.70
	Beijing (after 2008 Olympic games) [d]	5.02	11.00	4.40	11.70	32.20
	Guangzhou [e]	46.4	69.3	17.7	79.2	n/c

[a] North *et al.* (2017); [b] Miller *et al.* (2011); [c] Aguilera *et al.* (2009); [d] Liu *et al.* (2009); [e] Wang *et al.* (2002). n/c = not calculated or reported by the study

Birds were not exposed on sampling and measurement days for logistical and safety reasons.

4.3.2 Biomarkers

4.3.2.1 Morphometric measurements

The birds were weighed and measured on the day they were uniquely banded and allocated to the two groups (pre-adaptation), the day before the exposure started (d0), mid-exposure (d15) and at the termination of the experiment (d30). Head-bill length (base of the skull to the tip of the bill) and tarsal length were measured using digital calipers (± 0.01 mm). Wing chord, the length of the

wing from carpal joint to the end of the longest primary flight feathers, was measured using a modified ruler (± 1 mm), and body mass, using a 300 g Pesola® spring scale (± 1 g).

An indication of body condition, the Scaled Mass Index (SMI, Peig and Green (2009)) can be calculated using head-bill length, tarsal length or wing chord to normalize mass by differences in size. Peig and Green (2009) recommend the choice of the length variable with the strongest correlation with mass on a log-log scale. For this population, tarsal length, a familiar measurement less prone to error, overgrowth, or damage during captivity, was the best measure of size. Scaled Mass Index was calculated as follows: $SMI_i = M_i [L_0/L_i]^{bSMA}$; where b_{SMA} = *slope/Pearson's r*; where L_0, the reference length, is the mean tarsal length for all birds at the beginning of the experiment; M_i and L_i are the mass and length of the individual *i*; and b_{SMA}, the scaling exponent, is calculated as the slope of the regression line between the natural logs of mass and tarsal length, divided by the correlation coefficient. For this population of adult starlings, L_0 = 30.11 mm, and b_{SMA} = 2.992.

4.3.2.2 Immunotoxicology

The immunotoxic effects of vehicle emissions were investigated though testing the acquired branches of the immune responses; cell-mediated (T-cell) and humoral (B-cell) immunity. Cell mediated immunity was assessed using the phytohaemagglutinin (PHA) skin test, a widely used method of measuring T-cell response in wildlife, including birds (Smits *et al.*, 1999, Tella *et al.*, 2008). The method is described in Smits *et al.* (1999). Briefly, 30 µL of 1 mg/mL phytohaemagglutinin (PHA-M, Sigma Aldrich Inc., St Louis, MO, USA) in sterile phosphate-buffered saline (pH 7.4) was injected subcutaneously in the left wing-web of all control and exposed birds, on day 15 of exposure. The thickness of the wing web was calculated as the average of four measurements, before injection and 24 h (±1 h) later using a spring-loaded caliper (The Dyer Company, Lancaster, PA, USA). The difference between pre- and post-injection skin thickness provides a measure of T-cell mediated inflammatory response (Smits *et al.*, 1999). The same investigator measured both pre- and post-injection skin thickness.

The effects of BTEX exposure on the antibody (B-cell) response was assessed by vaccination against Newcastle disease virus (NDV). Commercial poultry eyedrop vaccine (Newcastle-Bronchitis vaccine, B1 type, B1 strain, Massachusetts and Connecticut types, live virus; Merial

Canada Inc.) was reconstituted using 20 mL sterile phosphate-buffered saline as diluent for 1000 dose vial, permitting the recommended dose to be administered in 20 µL instead of the customary 30 µL. Control and exposed birds were vaccinated mid-experiment and boosted with fresh vaccine two weeks later. Blood drawn at the beginning and end of the experiment was placed on ice until it could be centrifuged, and the serum frozen at -80°C. Pre- and post-vaccination antibody titers were measured using competitive ELISA (ID Screen® Newcastle Disease Competition, IDvet, Grabels, France), results read with a spectrophotometer (SpectraMax Plus384 Molecular Devices, Sunnyvale, CA, USA), reported as percent inhibition. Thirty-seven of the 44 birds had anti-NDV antibody titers below 30% inhibition one-week after the booster vaccination, a threshold below which is considered a negative response in poultry. However, since other avian studies have found similar low anti-NDV titers (Toro *et al.*, 2005, Scott *et al.*, 2013), and even 'inadequate' titers may be protective from disease (Chulan *et al.*, 1982), we felt it was justifiable to compare the change in titers (post-vaccination titer – pre-vaccination titer) between control and exposed birds directly.

4.3.2.3 Thyroid hormones & histology

Plasma total thyroxine (TT4), total triiodothyronine (TT3), and free T4 (fT4) were measured using solid phase, competitive enzyme-linked immunoassay (MP Biomedicals LLC), according to manufacturer protocols. Sample sizes were limited by the total volume of plasma required and varied among the tests as sample ran out. Samples and standards were run in duplicate for all thyroid assays. Four columns were run at a time for the TT4 assay, according to the manufacturer recommendations. The coefficient of variation (CV) among assays was 2.2%. All samples were run in a single assay for TT3 and fT4.

Thyroids were collected from each bird within 2h of euthanasia, fixed in formalin for routine histological processing and staining with hematoxylin and eosin. The thyroid sections were evaluated as described in Fernie *et al.* (2016) (Figure 4-2), using a CX31 microscope at 40x magnification, with DP72 camera and cellSens Standard image management software (all from Olympus Corporation©). For each bird, two randomly selected images were captured at 40x magnification. The total number of follicles on each 40x image was counted as a proxy for follicular size. The percentage of follicles containing endocytic vacuoles, or 'scalloping', was calculated as an indication of follicular cell activity (Lee *et al.*, 2016). Ten complete follicles were

randomly selected per image, and the epithelial cell height (ECH, µm) was averaged based on four measurements, one at approximately each of the four poles. The area (µm^2) and perimeter (µm) of the colloid contained within each follicle was recorded, and the ratio of colloid perimeter to ECH—indicating thyroid gland activation (Fernie *et al.*, 2016)—calculated. The results (two sections per bird, ten follicles per section) were averaged for each bird and expressed as the mean ECH, colloid area and ratio of colloid perimeter to ECH.

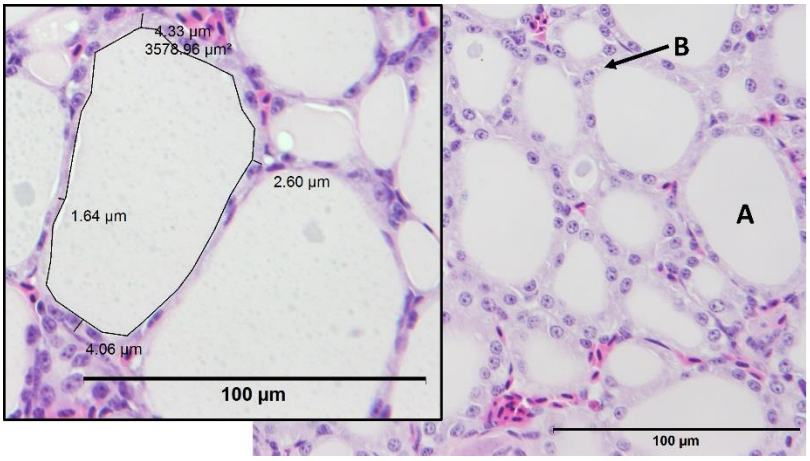

Figure 4-2. Section of a starling thyroid taken at 40x magnification. The colloid (A) area and perimeter, and the epithelial cell height (B) were measured as shown by the inset image (left).

4.3.2.4 Scaled organ mass indices

Relative organ sizes were calculated in a similar manner to scaled mass index, using the modification reported by Rodríguez-Estival *et al.* (2015). The b_{SMA} values used to calculate the scaled liver mass index (SLMI), scaled spleen mass index (SSMI) and scaled testes mass index (STMI) were 4.69, -20.70 and 62.85, respectively. The reference length used to calculate these indices was the same as that used for the scaled mass index (30.11 mm).

4.3.2.5 Hepatic detoxification

The activity of the cytochrome P450-1A1 biotransformation enzyme system was assessed by measuring hepatic 7-ethoxyresorufin-*O*-deethylase (EROD), using standard methods (Nilsen *et al.*, 1998), with slight modifications detailed in North *et al.* (2017). Samples, standards, blanks and pooled (positive control) samples were run in triplicate. The lowest concentration resorufin standard was removed, and an additional high-concentration (10 µM) standard was added to ensure

the generally high-activity of observed in starling samples in previous years were all within the standard curve. The incubation time was reduced to 10 minutes at 40℃ to further reduce the activity to within the curve. Protein concentrations were measured using a standard kit (Thermo Scientific Pierce™ BCA Protein Assay Kit), and EROD activity is reported as picomol resorufin per milligram protein per minute. Intraplate CV between replicate samples were 1.5%–2.0% and 0.5%–0.9%, and inter-plate CV was 7.6% and 1.9% for EROD and protein, respectively.

4.3.2.6 Oxidative stress

Two different antioxidant systems were measured to provide an indication of oxidative stress levels experienced by the starlings exposed to vehicle exhaust. Total and oxidized glutathione (tGSH and oxGSH, respectively) and thiobarbituric acid-reactive substances (TBARS) were measured in liver and testes, using the methods and modifications described in North *et al.* (2017). The protocol for oxGSH was modified slightly, adding 20 µL of triethanolamine instead of NaOH to neutralize excess vinylpyridine and bring pH to 6–7. TBARS samples were run in duplicate or triplicate, standards in duplicate. Coefficient of variation (CV, mean ± SD) among replicates and repeat reads were 4.29±2.45% (liver) and 4.82±1.91% (testes). For total and oxidized GSH, standards in duplicate and samples in triplicate, a maximum of six columns were analyzed at a time to optimize results. A standard curve was acceptable if $R \geq 0.996$. Clearly outlying data points were masked, provided that at least two replicates remained. The CV between the remaining replicates for tGSH was 2.14±1.40% for liver and 1.94±1.16% for testes, and for oxGSH, it was 1.21±2.06% (liver) and 0.81±0.42% (testes).

Reduced glutathione (rGSH) is calculated as total glutathione (tGSH) minus oxidized glutathione (oxGSH). Glutathione disulfide (GSSG) is equal to half the oxGSH concentration. We report tGSH, the ratio of rGSH to GSSG, and TBARS.

4.3.3 Statistical analysis

The efficiency of randomization was checked by running a preliminary Student's *t*-test for mass, tarsal and bill length, and body condition for the two groups. All responses were tested for normal distribution and homogeneity of variance using Q-Q plots and Levene's tests, respectively; SSMI (spleen), NDV antibodies, thyroid histology, and testes oxidative stress responses were

transformed using natural logarithm (ln). Outlying data were assessed and deleted on a case-by-case basis, where error was suspected. Associations between responses were assessed using Spearman's rho, with results expressing the strength of the correlation (r_S) and *p*-value. Change in scaled mass index during adaptation and exposure was analyzed using repeated measures ANOVA with a Greenhouse-Geisser correction for violation of sphericity and Fishers Least Significant Differences post hoc comparison; we report $F(df_{time}, df_{error}) = F$-value, $p = p$-value. Responses were analyzed using generalized linear models (GLM) with treatment group (exposed or control) and sex (male or female) included as fixed factors. Where sex was significant, the interaction of group and sex was included in the model. Results are expressed as the parameter estimate (B), 95% Wald confidence interval (95% CI: low, high) and significance (*p*-value). The effect of multiple comparisons on GLM results was checked by running a Students' *t*-test of the effect of exposure group on all responses together, and comparing the results to those from the GLM. While *t*-test results were marginally more conservative (larger *p*-values), there was no difference in which responses were significant, and therefore GLM results were regarded as true. The two-tailed significance was taken at $p<0.05$. All analyses were performed using IBM SPSS Statistics 24.0 software.

4.4 Results

4.4.1 Morphometric measurements

There were 28 birds in the control group and 26 birds in the exposed group; 19 females and 35 males were divided between groups. Male starlings were 4–5.7 g heavier than females throughout captivity. Both groups gained mass during adaptation (mass change, mean ± standard error of the mean (SEM): control, 7±1 g and exposed, 5±1 g), and lost mass during the exposure period (control, -4±1 g; exposed, -3±1 g, respectively). Change in mass (loss or gain) during exposure was not significantly different between groups or sexes.

Scaled mass index was not different between male and female birds; therefore, sex was not included in statistical models. The different time points had a significant effect on SMI ($F(2.401, 122.457)=44.121$, $p<0.0001$); likewise, the interaction between time and exposure group had a significant, but smaller effect on SMI ($F=3.436$, $p=0.028$). SMI was lowest before adaptation, and

peaked at the end of adaptation, before exposure. Group is only significant mid-exposure (B=-4.428; 95% CI: -8.163, -0.693; p=0.020), with exposed birds in better condition than controls. Relative organ masses were not different between groups or sexes. Scaled and organ mass indices are shown in Table 4-2.

Table 4-2. Scaled mass and organ mass indices of adult starlings exposed to vehicle exhaust, compared to controls [a]

		Control		Exposed		*p*-value
		n	**Mean ± SEM**	***n***	**Mean ± SEM**	
SMI	Pre-adaptation	31	81.10 ± 1.48	30	81.32 ± 0.85	0.899
	Post-adaptation	27	86.53 ± 1.47	27	87.57 ± 1.08	0.559
	Mid-exposure	**27**	**83.32 ± 1.56**	**27**	**87.75 ± 1.15**	**0.020**
	Post-exposure	27	82.17 ± 1.71	27	84.46 ± 0.97	0.237
	Δ during exposure	27	-4.36 ± 1.01	27	-3.12 ± 0.69	0.303
Organ mass indices	SLMI (liver)	28	2.87 ± 0.12	27	2.80 ± 0.08	0.640
	SSMI (spleen)*	27	0.13 ± 0.01	25	0.10 ± 0.01	0.130
	STMI (testes) *	18	0.22 ± 0.08	15	0.28 ± 0.11	0.836

[a] Units of grams; mean ± SEM. Significant results are shown in bold. Asterisks indicate *p*-value calculated using natural log-transformed data

4.4.2 Immune (T- and B-cell) responses

Considering the B-cell, antibody response, approximately 30% of the birds (13/44) did not seroconvert (less than 1% change after vaccination), with male and female, control and exposed birds represented equally. Neither exposure group nor sex were significant predictors of antibody response to vaccination against Newcastle disease virus. Skin thickness change in response to PHA stimulation was significantly affected by group (B=0.287; 95% CI: 0.164, 0.410; p<0.0001), with exposed birds having a smaller T-cell response than controls (Table 4-3). Neither sex nor interaction between group and sex were significant.

Table 4-3. Immune responses of starlings exposed to vehicle exhaust, compared to control birds [a]

	Control		Exposed		*p*-value
	n	**Mean ± SEM**	***n***	**Mean ± SEM**	
PHA (mm)	**28**	**0.45 ± 0.05**	**27**	**0.17 ± 0.04**	**<0.0001**
ΔNDV ab [b*]	18	9.05 ± 2.36	15	9.39 ± 3.31	0.687

[a] Significant results are shown in bold. [b] Antibody response to vaccination against NDV. Asterisks indicate *p*-value calculated using natural log-transformed data

4.4.3 Thyroid hormones and histology

Thyroid hormone and histology results are shown in Table 4-4. Neither group nor sex were significant for TT3 or fT4. Total T4 was significantly affected by group and group-by-sex (p=0.042 and 0.043, respectively), but not sex (p=0.128), with exposed males having lower TT4 concentrations than control males and females from both groups (B=6.438; 95%CI: 2.674, 10.293; p=0.001, Figure 4-3). None of the hormone levels were significantly correlated with any of the histological measurements.

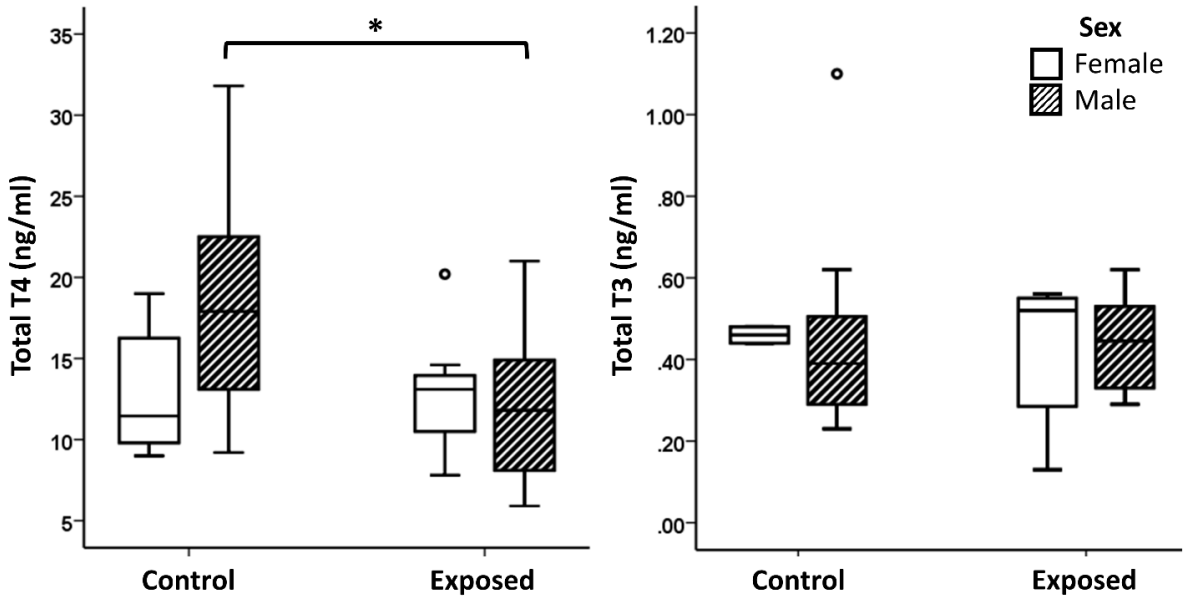

Figure 4-3. Total thyroxine (TT4) and triiodothyronine (TT3) concentrations in control and exposed European starlings, stratified by sex. * significant difference (p<0.05)

The number of follicles counted per high-power field (40x magnification) was correlated with colloid area, perimeter, and the ratio of perimeter to ECH (r_S=-0.825, =-0.854, and -0.603, respectively, p<0.0001, n=51), suggesting that the simple method of counting follicles using a light microscope may provide a reliable substitute for the other, more software-intensive methods. Epithelial cell height, colloid area, the ratio of colloid perimeter to ECH, and the percentage of follicles containing vacuoles were unrelated to group and sex (p>0.226).

Table 4-4. Thyroid histology and plasma hormones of European starlings exposed to vehicle exhaust, compared to control birds [a]

		Control		Exposed		*p*-value
		n	**Mean ± SEM**	***n***	**Mean ± SEM**	
TT3 (ng/ml)		12	0.40 ± 0.04	17	0.43 ± 0.03	0.571
fT4 (pg/ml)		13	3.07 ± 0.72	14	3.14 ± 0.48	0.936
# thyroid follicles [b*]		27	184.85 ± 18.32	25	173.41 ± 11.46	0.945
% vacuoles [c*]		26	19.28 ± 2.40	25	28.58 ± 4.00	0.226
ECH (µm) [d*]		26	3.56 ± 0.14	25	3.67 ± 0.19	0.782
Colloid area (μm^2) [*]		26	996.57 ± 108.63	25	865.93 ± 85.71	0.705
Perimeter:ECH [e*]		26	32.37 ± 2.25	25	30.52 ± 2.10	0.616
TT4 (ng/ml)	**Males**	**13**	**18.51 ± 1.88**	**13**	**12.07 ± 1.22**	**0.003**
	Females	8	12.88 ± 1.33	7	12.86 ± 1.53	0.992

[a] Significant responses are shown in bold. Where sex is significant, responses shown for males and females separately. [b] Number of thyroid follicles per 40x field. [c] Percentage of follicles in 40x field that contain visible vacuoles. [d] Epithelial cell height. [e] Ratio of colloid perimeter to ECH. Asterisks indicate p-value calculated using natural log-transformed data

The wide variation in thyroid histology among these starlings is shown in Figure 4-4.

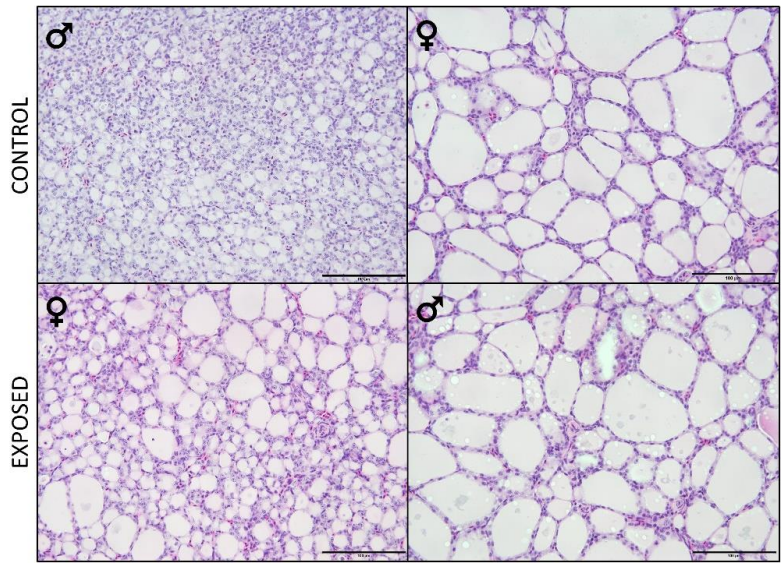

Figure 4-4. Photographs of thyroids with least (left) and greatest (right) colloid content for control (above) and exposed (below) starlings, demonstrating the extreme variation in thyroid histology that was unrelated to exposure to vehicle exhaust. Scale bar = 100 µm.

4.4.4 Liver EROD and oxidative stress

The EROD and oxidative stress responses are shown in Table 4-5. Exposed birds had lower tGSH than control birds (B=0.631; 95%CI: 0.004, 1.258; p=0.048). Sex, and group-by-sex were significant for liver TBARS (p=0.004 and 0.40, respectively), but not group (p=0.281), with control females showing higher lipid peroxidation than exposed females, and males from both groups (B=5.803; 95%CI: 0.272, 11.333; p=0.040). Neither group nor sex were significant for EROD activity (p>0.163) or the ratio of reduced GSH to GSSG in liver (p>0.509). Exposure was not significant for testes TBARS, tGSH, or rGSH:GSSG (p>0.642).

Table 4-5. EROD (liver) and oxidative stress responses (liver and testes) of adult starlings exposed to vehicle exhaust compared to control birds [a]

			Control		Exposed		*p*-value
			n	**Mean ± SEM**	***n***	**Mean ± SEM**	
Liver	EROD [b]		27	156.95 ± 10.38	27	150.66 ± 7.25	0.590
	TBARS [c]	Males	19	18.81 ± 1.37	15	20.19 ± 1.02	0.427
		Females	**7**	**25.77 ± 0.77**	**8**	**21.35 ± 1.47**	**0.006**
	tGSH [d]		**26**	**5.33 ± 0.22**	**24**	**4.70 ± 0.24**	**0.048**
	rGSH:GSSG [*]		26	23.30 ± 3.47	23	25.85 ± 4.30	0.672
Testes	TBARS [c,*]		7	10.86 ± 0.71	3	10.20 ± 0.29	0.642
	tGSH [d,*]		8	5.96 ± 0.29	3	6.16 ± 0.44	0.650
	rGSH:GSSG [*]		8	22.85 ± 2.37	3	21.94 ± 2.30	0.893

[a] Significant responses are shown in bold. [b] pmol/mg/min, [c] nmol/g, [d] μmol/g. Asterisks indicate *p*-value calculated using natural log-transformed data.

4.5 Discussion

This study demonstrates that exposure to traffic-source pollutants commonly found in large, more heavily-polluted urban areas has measurable effects on adult European starlings, with potential for population-level consequences. Exposed birds had suppressed T-cell mediated immune response, lower thyroxine and reduced levels of the major antioxidant, glutathione. The most sensitive of the biomarkers we examined, and the direction of change for various biological or physiological responses, are consistent with other studies investigating the effects of similar mixtures of air contaminants, despite concentrations in this study being 10–100-fold lower.

The T-cell suppression was also seen in tree swallow nestlings naturally exposed to oil sands tailings rich in volatile, petroleum-derived compounds (Cruz-Martinez *et al.*, 2015a), and in wild American kestrels experimentally exposed to a mixture of benzene and toluene (Olsgard *et al.*, 2008). While Olsgard *et al.* (2008) used a different method, measuring delayed-type hypersensitivity rather than PHA response, both methods depend upon cell-mediated immunity. In this study (Olsgard *et al.*, 2008), all the birds exposed to the benzene/toluene mixture showed this suppression, even at the lowest, 'environmentally relevant' rate, which was 20–150x higher

than in our study (0.1 ppm benzene, and 0.8 ppm toluene, versus our study peak values of approximately 4.37 ppb benzene and 5.52 ppb toluene during active exposure). Suppressed immunity could result in reduced resistance to parasitic infection, which commonly has a cost to free-living wild birds, particularly in developing nestlings that have immature immune responses (Eeva *et al.*, 1994, Gentes *et al.*, 2007b).

Decreased T4 in exposed birds corresponds with findings in American kestrels that were exposed to benzene, toluene, NO_2 and SO_2 (at 0.6, 1, 2 and 5.6 ppm, respectively) (Fernie *et al.*, 2016). American kestrels exposed to polybrominated diphenyl ethers and rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin show a similar pattern of responses: decreased T4 with unchanged T3 and thyroid histology (Brouwer *et al.*, 1998, Fernie *et al.*, 2005). The effects of petroleum compounds on thyroid hormones is highly varied, with exposure to some petroleum products having similar effects to those described in this study (Keil *et al.*, 2003), and exposure to others manifesting in completely different ways (Fowles *et al.*, 2016). There are several possible mechanisms of disruption in thyroid hormone metabolic pathways that may give the results observed in this study, one of which involves increased hepatic T4 glucuronidation and biliary secretion and the other being the binding of the chemical compound to transthyretin, a thyroid hormone-binding transport protein, that displaces T4 and leads to decreased serum T4 concentrations because of increased clearance (Brouwer *et al.*, 1998, Meerts *et al.*, 2000). With the critical hormones that control the metabolic rate, any imbalance may have diverse clinical manifestations, as varied as delayed or halted metamorphosis in amphibians (Hersikorn and Smits, 2011), compromised sexual maturation, and altered growth, development, metabolic rate and body condition of fish (Power *et al.*, 2001, Picard-Aitken *et al.*, 2007), and in the regulation of photoperiod-linked seasonal reproduction in starlings, quail, tree sparrows and hamsters (Dawson, 1993, Watanabe *et al.*, 2007). The sensitivity of T4 to vehicle emissions may play a role in certain human health effects, such as decreased birth-weights associated with exposure to traffic-related pollutants during pregnancy (Aguilera *et al.*, 2009) and other diverse, endocrine-related effects (Bolden *et al.*, 2015). The specific sensitivity of male birds to exposure may be explained by behavioral or seasonal changes related to other endocrinological cues; however, other avian toxicological studies have shown reduction in T4 levels in breeding male birds, after exposure to diverse toxicants (e.g., brominated flame retardants (Marteinson *et al.*, 2011)).

Air contaminants, including benzene, NO_2 and SO_2, have been implicated as causing oxidative stress via various mechanisms (Livingstone, 2001). Glutathione levels were reduced in exposed birds, exhibiting the same sensitivity and direction of change as observed in field studies on starlings (North *et al.*, 2017), and in total antioxidant capacity in urban house sparrows (Herrera-Dueñas *et al.*, 2014). The lack of evidence of cellular lipid peroxidation or altered GSH:GSSG ratios indicates that the exposed birds, while experiencing oxidative stress, were within their capacity to cope and maintain cellular antioxidant homeostasis.

Because exposure to benzene has been shown to increase thyroid hormones and glutathione (Bahadar *et al.*, 2014), this suggests that either other BTEX compounds, or other co-contaminants such as PAHs are responsible for the decreases in these biomarkers observed in this study. T-cell immunosuppression is consistent with many studies on benzene exposure, despite widely varying doses (Bahadar *et al.*, 2014).

Body condition, as calculated using the scaled mass index, was not significant for sex or the interaction between group and sex at any time, demonstrating that for adult starlings SMI indirectly corrects for sex-related differences in size and mass, enabling the direct comparison between groups without accounting for sex. This is useful when sex is harder to differentiate, such as for juvenile birds or adults not in breeding plumage. The loss in body condition in both the control and the exposed birds during exposure is likely due to a combination of factors specific to that period, including investigator presence, the noise of the truck and fans, and altered airflow in the enclosures, rather than toxicity.

After vaccinations and boosters with NDV vaccine, both groups demonstrated antibody responses that would be considered inadequate by commercial poultry standards. However, several publications have shown that these antibody cut-offs may not be applicable for interpreting NDV immunity status of wild birds (Toro *et al.*, 2005, Scott *et al.*, 2013). Nonetheless, even when accepting low antibody titers as real, there was no difference in the antibody response to vaccination between control and exposed birds.

Because of the earlier information providing evidence that the PAH concentrations in the emissions of an idling, modern gasoline engine would likely have been very low, PAHs were not

measured in this study. Because PAHs were not measured, we cannot rule out their contributions to some of the health effects observed in this study.

With this investigation, we show that contaminants found in vehicle exhaust emissions commonly found in large urban areas can have real, measurable effects on some key endocrine, immune, and antioxidant systems in wild birds. While the changes were subtle, they provide a warning that chronic exposure to environmental levels of these pollutants may have cumulative health effects, particularly for developing life stages that may be more vulnerable to endocrine disruption.

Chapter Five: **General Discussion and Conclusions**

5.1 General Discussion

Public interest is essential to receive ongoing societal and financial support for research, and to generate such interest it is important to provide information that can be related to people's own health, in the context of the broader ecosystem. Some effects of air pollution that may be detrimental to ecosystems, such as decreasing species diversity and disrupting population structure, may occur at levels of exposure not demonstrated to affect human health. Parallel human and ecological indicators allow a monitoring program to provide an integrated story, where all stakeholders can perceive some benefits of supporting the program. According to Burger and Gochfeld (1999), while the primary bioindicator of ecosystem health may be a single species, to give a complete and useful picture, it is ideal that a wide range of indicators also be measured to assess community structure, species diversity and other aspects of biological integrity, as well as abiotic components such as air, water or soil.

This project addresses a small part of this process, adding a piece of ecotoxicological knowledge to the patchwork quilt of epidemiological and classic toxicological evidence against air pollution. This work uses a combination of two study designs—a cross-sectional field study, with sampling sites chosen based on exposure and outcomes measured in groups of sibling starlings from these sites, followed by experimental exposure of wild-caught, adult starlings to confirm the sensitivity of the biomarkers used in the field study. One difficult aspect of studying the toxicology of these air pollutants is that they do not leave tissue residues, making it impossible to determine the 'biological dose', or absorbed dose, relying on the assumption that measured environmental contamination levels represent the true biological exposures experienced by the birds.

5.1.1 Calgary-specific conclusions

While Calgary has remarkably good air quality considering its population size, in some neighbourhoods traffic-related air pollutants are present in concentrations that may have long-term health implications. None exceed regulatory thresholds, but for BTEX we recommend further studies into whether regulated thresholds are low enough to protect our health. The Calgary Region Airshed Zone has made considerable progress with educational programs like the 'Idle Free schools' campaign, and we wish to encourage them to continue their excellent work. Reducing

traffic volumes by promoting carpooling, the use of public transport, and cycling to work are all essential, as well as the reduction of emissions at the individual vehicle level.

It has been shown that the concentrations of many air pollutants (e.g., NO_2 and VOCs) are higher during winter months, when local weather conditions promote the accumulation of pollution closer to the ground (temperature inversions), people tend to drive rather than walk, and heating systems are turned on. This is no different in Calgary (Bertazzon *et al.*, 2015). Therefore, it must be acknowledged that our conclusion that Calgary is a relatively clean city is based only upon measurements taken when the city would be at its best.

5.1.2 Unmeasured confounders and co-contaminants

Studies have shown that noise (e.g., traffic noise), and to a lesser extent, light pollution, may have measurable effects on urban wildlife reproduction and behaviour (Brewer, 1974, Fuller *et al.*, 2007, Francis *et al.*, 2009, González-Oreja *et al.*, 2012, Newport *et al.*, 2014). We did not measure noise in this study; however, traffic noise has been shown to be correlated with traffic-source air pollution (Davies *et al.*, 2009, Morelli *et al.*, 2015). Several of the studies showing effects of noise on avian reproduction did not account for possible effects of exposure to air pollution. In this study, none of the sites were directly adjacent to major arterial highways, and traffic noise was not noticeable at the highest-pollution sites. During the experimental study, vehicle noise was constant for control and experimental birds; thus, we can confidently conclude that biological responses were caused by emissions, and not confounded by noise.

Other unmeasured confounders like approved pesticide application and disturbance of the nest sites by the public, were not accounted for. However, no major effects were observed in the nestlings, nest boxes were well-concealed and did not appear to be disturbed by the public in any of the productive sites, leading us to conclude that these factors were not important during this study.

The adult starlings trapped for use in the experimental study may have had underlying, unknown contamination from feeding on agricultural crops in the Okanagan Valley, British Columbia; however, this population of starlings is largely non-migratory, and since all birds were from a limited geographic region, it is likely that all individuals would have similar contaminant burdens.

If these birds were previously exposed to pesticides, it is possible that they would demonstrate altered immune responses, hepatic detoxification, thyroid hormones and histology, and symptoms of oxidative stress (Bishop, 1998, Abdollahi *et al.*, 2004), among other changes. The rigorous randomization and month-long adaptation period should compensate for these confounding influences, but it could be said that there was no truly clean control group of birds in this study. None of the modern, approved pesticides leave detectable tissue residues, and the cost of testing all birds for unknown compounds would be prohibitive; however, in future studies it may be beneficial to retain tissue samples from the birds to test for heavy metals or brominated flame retardants if there is reason to suspect contamination. Ultimately, it would be better to select birds from areas where contamination with these contaminants is unlikely.

5.1.3 Which pollutant(s) is(are) responsible: BTEX, PAHs, or NO_2?

Does it matter? Yes!

Without knowing which pollutants are to blame for adverse health effects, it is impossible to report the direct cause-effect linkages necessary to compel people and Governments to alter their behaviour.

If traffic-related air pollution has measurable health effects in wildlife or people, then we need to find ways of reducing these emissions. For engineers to modify vehicles to reduce their emissions, they need to know which compounds they want to remove, since that will affect the chemistry behind the improved technology. Many epidemiological studies investigating adverse health effects of air pollution have used NO_2 as a proxy for other traffic-related emissions, since these contaminants share a common source, making them (mostly) highly correlated (Brook *et al.*, 2007). From a toxicology perspective, it is difficult to set up realistic experimental trials for pollutant mixtures like VOCs or PAHs, where getting the appropriate concentrations of numerous different compounds for prolonged exposures is far more difficult than setting up single-compound exposure trials, with e.g., NO_2, or benzene, or toluene.

The consequences of focussing on proxy pollutants for epidemiological studies and regulatory monitoring are not unfounded, and have led to reduced emissions from improved, cleaner fuels and more efficient vehicles with better catalytic converters. In the western world, these

technological advances have served to improve air quality in general, reducing ambient levels of many of the pollutants associated with vehicle emissions. However, the reduction in pollution levels is not be equal for all contaminants, and it is possible that the dramatic reduction in ambient NO_2 (>60% in the United States between 1980 and 2015) may expose health effects linked to pollutants with more moderate reductions in emissions of VOCs (54% reduction since 1980), or particulate matter (57% reduction) (U.S. EPA, 2017). Unfortunately, the U.S. EPA does not report emissions of PAHs, or air quality trends for PAHs, VOCs or BTEX, more specifically, so it is difficult to compare their trends to that of NO_2.

Bard *et al.* (2014) provide evidence that in France, for cases of myocardial infarction, benzene was a more important risk factor than NO_2, PM_{10}, O_3 or CO. While this is only one study, it highlights the fact that if the health effects of a wider range of pollutants is not considered, we risk misidentifying the true culprit(s) for air pollution-related adverse health effects.

5.1.4 Regulation ramblings

Many of the improvements in air quality in developed economies may be due to stricter regulation of fuel- and vehicle emission standards and a shift away from coal-fire power plants, countering the effects of population and economic growth. Depending on the regulatory agency, there may be different priorities and approaches to controlling air pollution. Concerning traffic-related air pollution, Europe and North America have historically had very different priorities based on differing concerns. When controlling vehicle emissions, vehicle manufacturers until now have had to choose between fuel economy, power and emissions. Additionally, there is the pay-off between CO_2 and NO_x emissions—the U.S. EPA has stringent regulations controlling CO and NO_x, direct threats to human health through the formation of acid rain and ozone which is a respiratory irritant. The consequence of their enforcement of these regulations is the increased production of CO_2 during redox reactions in catalytic converters, a non-toxic, yet known greenhouse gas contributing to climate change. The EU is more concerned about climate change and therefore has greater emphasis on fuel efficiency of vehicles and the production of ultra-clean fuels to reduce all emissions, but CO_2 in particular. The U.S. EPA is now implementing a national program for greenhouse gas emissions and fuel economy standards, focussing on 2012–2016 model cars as part of phase 1, and 2017–2025 model cars for phase 2 of the program (U.S. EPA, 2016c).

The petrol-versus-diesel debate rages on, with the arguments against diesel citing the high particulate matter and NO_x emissions from those vehicles compared to petrol-burning cars (e.g., Transport & Environment (2016)). There are merits and weaknesses for both fuels, with diesel cars being more duel-efficient overall, warming up more quickly, and producing less hydrocarbons and CO than petrol cars, but producing substantially more PM and NO_x (Anon (unkn.), http://www.air-quality.org.uk/26.php). Ultimately, at some point we will reach a ceiling in our ability to make hydrocarbon-based transport any cleaner, with modified commuter habits, alternative transport options, and cleaner technologies like electric vehicles leading the way from that point on.

Part of controlling emissions requires the decoupling of global economic growth (as GDP) from emissions, something that has been achieved by OECD (Organisation for Economic Cooperation and Development) countries (OECD Environment Directorate, 2008), and the United States (U.S. EPA, 2017). While developed countries are managing to achieve economic growth while controlling emissions, the cost of regulating and controlling emissions is beyond the budget of most developing, newly-industrialized nations, meaning that a large portion of the global population are still affected by air quality that is detrimental to their health (WHO, 2016). Additionally, the consequences of controlling primary pollutants at the source may result in an increase in secondary pollutants (e.g., particulate matter) and long-range transport of these particles from polluting countries to cleaner countries, with the manifestation of new health-related concerns (Pandis *et al.*, 2016).

Some researchers propose that there may not be 'safe' thresholds for certain pollutants like PM and ozone, since health effects are observed even at relatively low pollution levels (Brunekreef and Holgate, 2002). If this is the case, then current methods of regulating air quality may not be adequate, since they are based on the premise that, below a certain threshold, the presence of these contaminants in the air does not pose a problem.

5.2 Research Objectives Revisited

This thesis has addressed the three research objectives using several different, complementary methods, as described in the previous chapters:

Chapter 2 verified that the method we chose to measure air pollution in the city of Calgary was reliable, cost-effective and suitable for our purpose. In Appendix D, the passive samplers proved adaptable for use in an experimental setting, although some drawbacks were also identified, reiterating the value of incorporating diverse methods to meet different objectives.

Biomarkers that were sensitive to exposure to ambient, urban air pollution were described for nestling European starlings in Chapter 3, in the context of confounding factors common to field research. Chapter 4 described sensitive responses in adult starlings experimentally exposed to vehicle exhaust at concentrations likely below those of many urban areas globally. Glutathione proved sensitive to exposure in both the field and experimental studies; clutch size, body condition and EROD were found to be sensitive to exposure in the field study, and thyroid hormones in the experimental study.

These measurable changes suggest that European starlings are sensitive to common urban air pollutants, although not to the point of eliciting clinical disease. Their natural abundance, tolerance of handling, adaptability and ability to cope with exposure to air pollutants makes starlings excellent sentinels of possible adverse effects in other wildlife and people. More research is needed to relate starlings' sensitivity to air pollutants to other common urban species including sparrows, pigeons, or small mammals, and what this evidence of endocrine and oxidative stress in starlings means for human residents. However, the evidence that exposure to relatively low levels of air pollution elicits hormonal changes should serve as a warning—since we still don't know the effects of chronic exposure, the effects on developing foetuses, and the likelihood that regulatory thresholds may not be sufficient to protect the vulnerable members of society.

5.3 Future Directions

It would be valuable to reassess the field study using LUR 2015 summer values for particulate matter, black carbon, etc. as exposure predictors on the nestling responses, since we know that the LUR 2015 corresponds very well with passive sampler data for NO_2 and BTEX. If these other contaminants show a different spatial distribution to NO_2 and BTEX, then it may be possible to tease out their effects on the starlings using data that has already been gathered.

Feathers have been banked from all birds in this study; once the feather corticosterone method can be optimised, it would be worthwhile to examine the stress response to exposure to air pollution. Similarly, organ samples have been preserved in formalin and should be analysed if possible.

In future field studies, based on our findings, the following are recommended as potential biomarkers:

- Thyroid hormones (total, and free, where serum volume is sufficient)
- Detailed histological analyses of target organs (liver, lung, thyroids)
- Other oxidative stress pathways
- Carotenoids, both as ornamentation during the breeding season, and serum carotenoid concentrations
- Gut microbiome

These include several potential biomarkers initially included in the research proposal, but which were excluded due to methodological problems, or time and financial constraints. These markers could be included in similar research in the future. For detail on each, please refer to the appendices and referenced literature.

If adult birds are trapped for experimental exposures, during necropsy, a portion of the liver and muscle tissue should be reserved for toxic residues associated with previous metal- and pesticide exposures. Since the birds in this study were trapped in an agricultural region with intensive fruit production, it is possible that some of the thyroid histological changes, for example, were related to prior exposure to toxicants. Knowing the history of the study subjects could provoke analyses for other contaminants in the tissues which would allow control for their confounding influences.

Study designs used in field toxicology can vary from fine-resolution, statistically-powerful studies that have many sites representing a range of exposures with minimal clustering, to classic two site studies (exposed and reference), with many subjects in each site. While the former allows greater sensitivity for detecting exposure thresholds, dose-response relationships, and may uncouple the relative contribution of confounders from that caused by the exposure of interest, the method is also logistically difficult, extremely time-consuming, expensive, and the statistical analyses

complex. If the exposures are low and the responses to exposure unknown or undetectable, then the benefits of this design are outweighed by the costs of its implementation.

The latter two-site design is easier to implement, and the responses linked to exposure easier to analyze. For these reasons, it may be the design of choice when initiating a study, to identify sensitive biomarkers that you may go on to test under other conditions, and for research into toxicology of specific industrial sites, spill sites, or other point-source exposures that don't occur at ecologically comparable reference sites. However, in some cases, it may not be possible to disentangle the effects of exposure from those of confounders inherent to the exposed and reference sites, e.g., unmeasured co-exposures, differences in microclimate, food quality or quantity, with pseudoreplication (are the responses of many individuals within a site truly independent?) being a major concern. The choice of design should be based on the objective of the study, pre-existing knowledge of the exposures of interest, balanced with the resources available to the investigator.

5.4 Benefits of a One-Health Approach to Air Quality Monitoring

Air pollution has been known to affect human health for centuries, with residents of ancient Rome recognising it as a nuisance and taking breaks from the foul odours of the city (Chen *et al.*, 2007). Since the industrial revolution, humans have had an increasing chemical footprint, with the effects of airborne industrial emissions on plant- and human health being noted as early as 1661 (Cowling, 1982).

Air pollution in all its forms (pesticides, polychlorinated biphenyls (PCBs), flame retardants, heavy metals, particulate matter, nitrates/nitrites, sulfates/sulfites, ozone, volatile organic compounds, etc.) is indiscriminate in its effects, targeting humans, animals, invertebrates and plants in equal measure. The chemistry of the compounds determines its persistence and transport, with some pollutants degrading to other forms within hours when exposed to sunlight (e.g., NO_x and VOCs reacting to form O_3), and others being capable of being transported across the world and deposited at the poles (e.g., PCBs).

Without accurate exposure data for every subject in an epidemiological study, it is extremely difficult to allocate symptoms to an individual pollutant. Many advanced modelling techniques are

becoming available to improve the estimation of exposure, including land use regression models that predict exposure based on proximity to, for example, major roads, industrial areas, and account for topography and predominate wind direction. Personal, badge-type air monitors also improve exposure-allocation, allowing the researchers to track study subjects' exposure to contaminants throughout their daily routine.

Studying wildlife species that may be more sensitive to airborne pollutants than the average human, provides an indication of pollution levels that may be eliciting effects in vulnerable populations. The additional benefit of being able to take invasive samples can help researchers understand the mechanisms of toxicity, which may translate to predicting or explaining some of the associations being detected during epidemiological studies.

Monitoring the health of plant life in regions affected by air pollutants remains critical, since they are known to be sensitive to many of these compounds, they are critical to life on earth, and there is no known way of predicting effects on plants based on those observed in animals. The effects of exposure on invertebrate populations is similarly critical, with so many species depending on them for food.

Quantifying the effects of exposure to airborne contaminants is therefore a quintessential One Health problem, which, if not treated as such, is unlikely to be concluded in a sustainable fashion. If regulatory thresholds are based on single-pollutant studies instead of mixtures, or on effects observed in laboratory species and epidemiological studies without regarding wildlife, plants or invertebrates, then effects will continue in vulnerable populations.

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APPENDIX A: ALBERTA CENTRE FOR TOXICOLOGY METHODS FOR ANALYSING PASSIVE SAMPLERS

These method summaries are included with permission from the Alberta Centre for Toxicology (ACFT), and include preparation, deployment, and methods of analysing Ogawa samplers (NO_2 and SO_2, Ogawa, USA) and 3M VOC samplers (Organic Vapor Diffusion Monitor 3500, 3M™, USA), that were used during the research detailed in this thesis. O_3 is included in the methods because it forms part of the ACFT methods, but is not relevant to this thesis. Sampler assembly is per manufacturer instructions and not included here.

A.1. Ogawa Air Sampler Method Summary - SO_2, NO_2 and O_3

Figure A-5. Ogawa passive samplers used to measure NO_2 and SO_2 in this study

A.1.1. Preparation and use procedure

To prepare the blank monitor to use:

- A blank monitor should be included with each set of samples to check for any contamination of the samples. The blank should be opened when the sample is opened.
- Remove a Sampler ID# label and place it on the "Passive Samplers – Log Sheet for Deployment" form enclosed with the monitor.

- Remove the monitor from the brown storage bottle and the re-sealable bag, and then immediately place back in the bag and in the bottle.
- Close the storage bottle with the lid provided.
- Store the blank monitor refrigerated (4°C) while other monitors are being deployed.

To prepare the monitor for use:

- Remove a Sampler ID# label and place it on the "Passive Samplers – Log Sheet for Deployment" form.
- Record the following information on the form "Passive Samplers – Log Sheet for Deployment":
    - Participant ID#
    - Sampling Start Date
    - Comments (e.g., temperature, relative humidity, rain, etc.)
- Remove the sampler from the re-sealable bag. Secure the sampler in the desired location.
    - As a personal monitor, it should be worn near the worker's breathing zone (e.g., front of the shirt collar).
    - As an area monitor, hang it away from walls, corners, table tops or other regions where air movement in the area may be limited.
- Replace the re-sealable bag in the brown storage bottle and tighten the cap securely. Retain it for later use.

To prepare the monitor at the end of sampling:

- Remove the sampler from the sampling location and place it in the re-sealable bag, then in the brown storage bottle, and tighten the cap securely.
- Record the Sampling End Date and Sampling End Time. Ensure the "Passive Samplers – Log Sheet for Deployment" form is filled out appropriately.
- Transport the monitor(s), the blank and the completed form to ACFT within 48 hours. Store the monitors in refrigerator (4°C) if delay is anticipated

A.1.2. Laboratory analysis

Adapted from: F14027 Ogawa Air Sampler Method Summary Version 140505

Introduction:

Alberta Centre for Toxicology (ACFT) purchases the Ambient Air Passive Air Samplers and Collection Pads for SO_2, NO_2, and O_3 from the Ogawa & Co. ACFT washes all the reusable sampler parts and assembles the pads into the samplers. Exposed samplers are analyzed at ACFT or RTI International (3040 Cornwallis Road, Bldg 6, RTP, NC 27709).

Air Sampler Extraction at (ACFT):

SO_2 Samplers: Each sampler is disassembled and the pad is removed from the holder. The pad is placed in a culture tube. A volume of 5 mL of extraction solvent (Type I water with 2 µg/mL HPO_4^{2-} as internal standard) is added to the tube using a Brinkman bottle top dispenser. The culture tube is then capped and inverted every 5 minutes for 30 minutes. After 30 minutes, 60 µL of 30% hydrogen peroxide is pipetted into the culture tube. The tube is inverted slowly every half an hour for 2 hours. Two mL of the extract is transferred into a Dionex IC vial, capped, and injected on the Dionex IC along with a set of calibrators and controls.

NO_2 Samplers: Each sampler is disassembled and the pad is removed from the holder. The pad is placed in a culture tube. A volume of 8 mL of extraction solvent (Type I water with 2 µg/mL HPO_4^{2-} as internal standard) is added to the tube using a Brinkman bottle top dispenser. The culture tube is capped and placed on a tube rocker to shake for 15 minutes. Approximately 1.5 mL of the extract is transferred into a Dionex IC vial, capped, and injected on the Dionex IC along with a set of calibrators and controls.

O_3 Samplers: Each sampler is disassembled and the two pads are removed from the holder. The pads are placed in a culture tube. A volume of 5 mL of extraction solvent (Type I water with 2 µg/mL HPO_4^{2-} as internal standard) is added to the tube using a Brinkman bottle top dispenser. The culture tube is capped and placed on a tube rocker to shake for 15 minutes. Approximately 1.5 mL of the extract is transferred into a Dionex IC vial, capped, and injected on the Dionex IC along with a set of calibrators and controls.

Analysis of Ogawa Air Sampler Extractions at ACFT:

Ion Chromatography in conjunction with chemical suppression and conductivity detection is used for the separation and determination of the anions in Passive Air Samplers. The system used is a Dionex DX500 Ion Chromatograph comprised of the following: a Dionex LC25 chromatography cven, a Dionex AS40 automated sampler, a Dionex CD20 conductivity detector, a Dionex GP40 gradient pump, as well as a Metrohm 753 suppressor module.

Separation of the anions is performed on a Dionex IonPac AS12A analytical column (4 x 200 mm) using a Dionex IonPac AG12A guard column (4 x 50 mm). The mobile phase consists of 2.7 mM sodium carbonate (Na_2CO_3) and 0.3 mM sodium bicarbonate ($NaHCO_3$), which is filtered through a 0.22 µm filter. 100 µL of sample is injected onto the IC column with a flow rate of 1.5 mL/min. Data is collected over an 11 minutes run time. The anions are identified according to their retention times and elution order. An internal calibration is used to determine the anion concentrations.

The anions detected are (in order of elution):

1. Nitrite (NO_2^-): for the NO_2 Passive Samplers
2. Nitrate (NO_3^-): for the O_3 Passive Samplers
3. Sulfate (SO_4^{2-}): for the SO_2 Passive Samplers

A.1.3. Data Analysis at ACFT

The results are collected as a concentration in µg/mL and converted into ng/sampler by multiplying the concentration measured by the amount of extraction solvent used in the extraction and a conversion factor of 1000 to give concentrations in ng/sampler.

- For SO_2, SO_4 concentration (ng/sampler) = SO_4 (µg/mL) * 5 (mL) * 1000
- For NO_2, NO_2 concentration (ng/sampler) = NO_2 (µg/mL) * 8 (mL) * 1000
- For O_3, NO_3 concentration (ng/sampler) = NO_3 (µg/mL) * 5 (mL) * 1000

LOQ: The limit of quantitation for this method is 0.1 µg/mL for Nitrite (NO_2^-), Nitrate (NO_3^-), and Sulfate (SO_4^{2-}). Value below 0.1 µg/mL will be reported as information only for research study.

Linearity: This method is linear up to 50 µg/mL for the anions. Dilutions are performed on any sample with a concentration greater than 50 µg/mL.

A.1.4. Data Analysis at RIT International

The equations for calculating concentration in ppb (by volume) are based on the following protocols from the Ogawa website www.ogawausa.com and they are attached as references with this summary:

1. NO, NO_2, NO_X AND SO_2 SAMPLING PROTOCOL USING THE OGAWA SAMPLER (PAGE 23, 28)
2. PROTOCOL FOR OZONE MEASUREMENT USING THE OZONE PASSIVE SAMPLER BADGE (PAGE 14)

From protocol 1 (page 23), SO_2 concentration (ppb) = $\alpha SO_2 * WSO_2 / t$
where:

- WSO_2 is the sulfate quantity (in ng) collected on the SO_2 sampler multiplying the molecular weight of SO_2 and divided by molecular weight of SO_4.
- $WSO_2 = WSO_4$ (in ng) * 64.062/96.06 (Molecular weight of SO_2 = 64.062 g/mole; Molecular weight of SO_4 = 96.06 g/mole)
- αSO_2 is the ppb concentration conversion coefficients (ppb-min/ng). At 20°C the value of $\alpha SO_2 = 39$.
- t is sample collection time in minutes.

From protocol 1 (page 28), NO_2 concentration (ppb) = $\alpha NO_2 * WNO_2 / t$
where:

- WNO_2 is the nitrite quantity (in ng) collected on the NO_2 sampler,
- αNO_2 is the ppb concentration conversion coefficients (ppb-min/ng). At 20°C the value of $\alpha NO_2 = 56$.
- t is sample collection time in minutes.

From protocol 2 (page 14), O_3 concentration (ppb) = (18.09) * 1000 * WNO_3 / t
where:

- WNO_3 is the nitrate quantity (in µg) collected on the O_3 sampler,
- 18.09 is the conversion coefficient from NO_3 to O_3. Derivation of this coefficient is detailed on page 14.
- "1000" is the conversion factor from ppm to ppb.
- t is sample collection time in minutes.

A.2. Volatile Organic Compounds (VOC) in Air Sampler Method Summary

3M^{TM} Organic Vapor Diffusion Monitor 3500 (Figure A-2)

Figure A-6. 3M^{TM} Organic Vapor Diffusion Monitor 3500 used for measuring VOC concentrations in this study

A.2.1. Preparation and use procedure

To prepare the blank monitor to use:

- A blank monitor should be included with each set of samples to check for any contamination of the samples. The blank should be opened when the sample is opened.
- Remove a Sampler ID# label and place it on the back of the monitor.
- Remove another Sampler ID# label and place it on the "Passive Samplers – Log Sheet for Deployment" form enclosed with the monitor.
- Remove the plastic ring and white film from the monitor. Immediately snap the elution cap (with plugs) onto the main monitor body. Be sure the two port plugs are secured.
- Return the blank monitor into the can and close the can with the plastic lid provided.
- Store the blank monitor refrigerated (4°C) while other monitors are being deployed.

To prepare the monitor for use:

- Remove a Sampler ID# label and place it on the back of the monitor.
- Remove another Sampler ID# label and place it on the "Passive Samplers – Log Sheet for Deployment" form enclosed with the monitor.
- Record the following information on the form "Passive Samplers – Log Sheet for Deployment":
    - Participant ID#
    - Sampling Start Date
    - Comments (e.g. temperature, relative humidity, rain, etc.)
- DO NOT remove the white film and plastic ring. Secure the monitor in the desired location.
- As a personal monitor, it should be worn near the worker's breathing zone (e.g. front of the shirt collar).
- As an area monitor, hang it away from walls, corners, table tops or other regions where air movement in the area may be limited.
- Close the can with the plastic lid provided. Retain it for later use.

To prepare the monitor at the end of sampling:

- Remove the plastic ring and white film from the monitor. Immediately snap the elution cap (with plugs) onto the main monitor body. Be sure the two port plugs are secured. Return the monitor to the can. Close the can with the plastic lid provided.
- Record the Sampling End Date and Sampling End Time. Ensure the "Passive Samplers – Log Sheet for Deployment" form is filled out appropriately.
- Transport the monitor(s), the blank and the completed form to ACFT within 48 hours. Store the monitors in refrigerator (4℃) if delay is anticipated.

A.2.2. Laboratory analysis

VOC Sample Extraction:

Each passive monitor is filled with 1.5 mL of carbon disulfide solvent and allowed to sit for 30 minutes. The carbon disulfide is then transferred from the badge into a glass vial and securely capped.

Analysis of VOCs:

The samples are injected onto a gas chromatograph (GC) for compound separation followed by detection by a mass spectrometer (MS). The system used is a Hewlett-Packard GC-6890/MS-5973. Separation is performed on an HP 19091V-402 capillary column, 25m x 200µm x 1.12µm at an oven temperature range of 40^{o}C - 140^{o}C. Total run time is 17.5 minutes and sample injection volume is 1µL.

The following compounds are detected:

- Hexane
- 3-Methylhexane
- Benzene
- Heptane
- Toluene
- Octane
- Ethylbenzene
- m,p-Xylenes
- Nonane
- o-Xylene
- n-Propylbenzene
- Decane
- Limonene
- n-Butylbenzene

A.2.3. Data analysis

Calculations: The GC/MS gives VOC concentrations in µg/mL. These concentrations are multiplied by the extraction volume of 1.5 mL and a conversion factor of 1000 to give concentrations in ng/badge.

The formula for converting ng/badge to µg/m^3 is

$$\text{Analyte } (\mu g/m^3) = \frac{\text{Analyte concentration (ng/badge) x 1000}}{\text{Time exposed (min) x Sampling Rate (mL/min)}}$$

Where Time exposed for a 7-day period = 10080 min

Compound	Sampling Rate from 3M Manufacturer (mL/min)	LOQ (ng/badge)	LOQ for 7-day Exposed Period (ug/m^3)
Hexane	32.0	150	0.47
Methylhexane	28.9	150	0.51
Benzene	35.5	150	0.42
Heptane	28.9	150	0.51
Toluene	31.4	150	0.47
Octane	26.6	150	0.56
Ethylbenzene	27.3	150	0.55
m,p-Xylenes	27.3	300	1.09
o-Xylene	27.3	150	0.55
Nonane	24.6	150	0.60
Decane	23.1	150	0.64
Limonene	21.9	150	0.68
N-Propylbenzene	24.6	150	0.60
N-Butylbenzene	22.4	150	0.66

LOQ: The limit of quantitation for this method is 0.2µg/mL for *m,p*-Xylenes and 0.1 µg/mL for the remaining compounds. Any value below 0.1µg/mL is reported as 0 µg/mL. The method detection limit for *m,p*-Xylenes is 300 ng/sampler and 150 ng/sampler for the remaining VOC's.

Linearity: This method is linear up to 50 µg/mL for all compounds. Dilutions are performed on any sample with a concentration greater than 50 µg/mL.

APPENDIX B: SUPPORTING INFORMATION (SI): EUROPEAN STARLINGS (*STURNUS VULGARIS*) AS SENTINELS OF URBAN AIR POLLUTION: A COMPREHENSIVE APPROACH FROM NON-INVASIVE TO POST MORTEM INVESTIGATION

B.1. Materials and Methods

Additional details on the Materials & Methods of specific sections.

B.1.1. Possible confounders: year, hatch date, weather

As with any field study, many factors may confound the interpretation of results. These were investigated and accounted for as far as possible by including the confounder or a proxy in the final analyses.

Nestling starlings were monitored for the summers of 2013 to 2015; the same investigator performed all measurements in 2013 (pilot study), while a different investigator (MN) conducted all aspects of the study for 2014 and 2015. Variation in environmental conditions and experimental methodology between years was accounted for by including year as a factor in all statistical analyses. Individual nestling responses from 2013 were excluded from analysis for this paper since they are more sensitive to operator differences, whereas reproductive responses are more robust to variation in investigator technique and therefore those data from 2013 were included.

The timing of reproduction can significantly affect many reproductive and morphological variables—it is critical for birds to nest at an optimal time to ensure sufficient quality food for their brood, weather that promotes offspring survival, as well as enough time for the chicks to fully mature to maximize migration and overwinter survival. The date of hatch (Julian date, or ordinal calendar date) was included as a covariate in all models to account for this source of variance.

The effect of weather on reproductive success and nestling growth was assessed using meteorological data archived by Environment Canada [1]. Daily meteorological data were extracted for May to July of 2013 to 2015 from the Calgary International Airport weather station. While these data are not precise for each location, they provide a good indication of the weather in Calgary for each date. Mean daily precipitation, total precipitation, mean minimum and maximum

daily temperature, mean daily diurnal temperature range (max - min), and mean temperature (mean of maximum and minimum temperatures) were calculated for the week before and the week after the hatch date for each nest. Total precipitation includes snow and rain which was calculated and reported by the weather station.

See Figures B-1 and B-2 for visual detail on the relationship between year, hatch date, precipitation and temperature.

B.1.2. Biological indicators and analytical methods

Hepatic EROD activity

The activity of cytochrome P4501A1 (CYP1A1) monooxygenase in the livers of nestling starlings exposed to ambient air pollution was determined by measuring 7-ethoxyresorufin-O-deethylase (EROD) activity, following the standard protocol described by Nilsen, et al. [2], with modifications to buffers and incubation time described below:

Frozen liver (0.2 g to 0.6 g) was hand-homogenized in homogenization buffer (2 ml, 0.1 M sodium phosphate, pH 7.4, 0.15 M KCl, 1 mM EDTA), transferred to 5 ml ultracentrifuge tubes, balanced and centrifuged at 9,000 g for 30 minutes at 4°C. The microsomal pellet was resuspended using resuspension buffer (1 µl buffer per 1 mg liver; 0.1 M sodium phosphate, pH 7.4, 0.15 M KCl, 1 mM EDTA, 1 mM DTT and 20 % (v/v) glycerol) after the second round of ultracentrifugation. Aliquots of resuspended microsomes were stored at -80°C (< 1 month), before being analyzed for protein concentration and EROD activity. Samples were pre-incubated for 5 minutes in black, 96-well plates in the dark at room temperature before adding NADPH, after which they were incubated for 18 minutes (2014) or 12 minutes (2015) at 40°C in the plate reader before stopping the reaction with acetonitrile. The final assay contained 20 µl sample (diluted to 1 µg/µl protein), 30 µl β-nicotinamide adenine dinucleotide phosphate (NADPH, 1 mM) and 150 µl 7-ER (2 µM) per well.

The microsomal protein concentration was measured using Pierce™ BCA Protein kit (Thermo Fisher Scientific Inc., Rockford, IL), following standard procedure and results were read in a spectrophotometric plate reader and SoftMax Pro software.

Lipid peroxidation and GSH redox status in liver

Minor adjustments were made in 2015 to improve efficiency of the analysis of tGSH and oxGSH, as follows: Working solutions were prepared as described in Rodríguez-Estival, et al. [3], and combined to form a reaction mix immediately prior to use. Reaction mix (200 µl of 0.24 mM NADPH, 0.5 mM 5,5'-dithiobis (2-nitrobenzoic acid), and 1 IU glutathione reductase/ml) was added to 40 µl sample in a 96-well plate, maintaining the same final concentrations in each well as in the published method. The reaction mix was added using a multichannel pipette, enabling five columns to be run at a time (two with standards and three with samples). Two additional low concentration standards were added to extend- and improve the fit of the standard curve in 2015, and the reaction measured using a spectrophotometric plate reader and SoftMax Pro reading as a kinetic assay for 5 minutes.

B.2. Reliability of Laboratory Results

The reliability of the laboratory tests used to determine hepatic EROD, tGSH and oxGSH was assured using the following quality controls:

- A standard curve was generated for every assay, using eight standards for each plate for EROD, four standards and one blank per plate for TBARS, and eight standards for tGSH and seven for oxGSH, which were each run five rows at a time (standards repeated for each five-row assay).
- A pooled, control sample was run on every plate for each year.
- All samples and standards were run in duplicate or triplicate (see Table B-1).
- In 2015, for EROD all samples could be run on a single plate, therefore eliminating inter-assay variation.
- The coefficient of variation (CV%) between duplicate samples, among assays and plates was calculated. For the standard curve, if the CV>15% for duplicate or triplicate standards, the values for the individual wells were checked and outlying points removed to obtain a standard curve with R>95%.

The large CV between years and between assays for oxGSH in 2014 prompted the exclusion of all 2014 oxGSH results (Table B-2).

TABLES

Table B-1. CV of the replicates for each sample (duplicate or triplicate), for 2014 & 2015

	%CV (mean ± SD) for replicate samples			
	tGSH	oxGSH	TBARS	EROD
2014	13.41 ± 11.83*	15.79 ± 15.41	4.00 ± 10.68	3.29 ± 2.44
2015	1.52 ± 1.16	1.68 ± 2.79	5.16 ± 6.61	3.01 ± 2.10

* raw data before removing samples with high variance

Table B-2. Inter-assay %CVs (mean ± SD) for standard curves (between years & plates)

TBARS	
mean CV	2.84
SD of CV	4.04
(range of CV)	0.32 - 9.98
tGSH [1]	
mean CV	31.41
SD of CV	41.65
(range of CV)	3.34 - 109.80
oxGSH [2]	
mean CV	250.90
SD of CV	543.90
(range of CV)	6.88 - 1476.45
EROD	
mean CV	14.58
SD of CV	13.06
(range of CV)	5.08 - 38.26 [3]

[1] raw data before removing samples with high variance. [2] data from 2014 was largely responsible for the large amount of variation. [3] note that the highest CV was found for low concentration standards

Table B-3. Volatile organic compounds measured using 3M Organic Vapor Monitors.

Volatile Organic Compounds (µg/m^3):	
Benzene	Integrated as BTEX (sum of the individual compound concentrations); highly correlated with all VOCs (p<0.0001) except octane, justifying its use as a proxy for VOC exposure
Toluene	
Ethylbenzene [a]	
m, p-Xylenes	
o-Xylene	
Hexane	
3-Methylhexane [ab]	
Heptane [a]	
Octane [ab]	
Nonane [ab]	
N-Propylbenzene [ab]	
Decane [ab]	
Limonene [ab]	
N-Butylbenzene [ab]	

[a] These were below the limit of detection (LOD) / limit of quantification (LOQ) for the analytical laboratory in the 2014 monitoring period. [b] Volatile organic compounds below LOD/LOQ in 2015.

Table B-4. WHO, U.S. EPA and Alberta regulatory thresholds for air contaminants measured in this study.

Compound	Exposure duration *	WHO Guidelines [a]	U.S. EPA NAAQS [b]		Alberta Ambient Air Quality Objectives [c]	This study (mean ± SD, 2013–2015)
			Primary (public health)	Secondary (public welfare)		
Nitrogen dioxide	1-hour	200 µg/m^3 (~ 105 ppbv) †	100 ppbv		300 µg/m^3 (159 ppbv)	4.64 ± 0.24 ppbv
	annual	40 µg/m^3 (~ 21 ppbv) †	53 ppbv	53 ppbv	45 µg/m^3 (24 ppbv)	
Sulphur dioxide	10-minute	500 µg/m^3 (~ 188 ppbv) †	75 ppbv	0.5 ppmv		0.31 ± 0.03 ppbv
	1-hour				450 µg/m^3 (172 ppbv)	
	3-hour	20 µg/m^3 (~ 7.5 ppbv) †				
	24-hour				125 µg/m^3 (48 ppbv)	
	30-day				30 µg/m^3 (11 ppbv)	
	annual				20 µg/m^3 (8 ppbv)	
Volatile organic compounds						
Benzene	1-hour				30 µg/m^3	0.25 ± 0.08 µg/m^3
	annual				3 µg/m^3	
Toluene	1-hour				1,880 µg/m^3	0.85 ± 0.36 µg/m^3
	24-hour				400 µg/m^3	
Ethylbenzene	1-hour				2,000 µg/m^3	0.14 ± 0.08 µg/m^3
Xylenes	1-hour				2,300 µg/m^3	0.75 ± 0.35 µg/m^3
	24-hour				700 µg/m^3	

[a] World Health Organization (WHO) Media center: Ambient (outdoor) air quality and health, fact sheet. Updated September 2016. URL: http://www.who.int/mediacentre/factsheets/fs313/en/. [b] U.S. EPA National Ambient Air Quality Standards (NAAQS), criteria air pollutants. Updated 20 December 2016. URL: https://www.epa.gov/criteria-air-pollutants/naaqs-table%20. [c] Alberta Ambient Air Quality Objectives and Guidelines Summary, document number: AEP, Air Policy, 2016, No. 2. Updated June 2016. ISBN 978-1-4601-2861-9 (PDF). * All values represent the mean concentration of that pollutant over the specified period. † Conversion between µg/m3 and ppbv (parts per billion, volume) for NO_2 and SO_2 based on conversion factors published by the UK Air Quality Archive (using the method for reporting data to the European Commission) (Nitrogen dioxide 1 ppbv = 1.9125 µg/m^3; Sulfur dioxide 1 ppbv = 2.6609 µg/m^3)

Table B-5. Correlations (Spearman's rho, r_s) among temperature (°C,), precipitation (mm), year & hatch date (HDJ), for nest-level data (2013–2015).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Year	r_s	1	-0.28	0.13	**-.442****	0.122	**-.443****	**-.324***	-0.095	**-.378****	**.385****	0.159	**.552****	**-.389****	0.157
	p		0.056	0.384	0.002	0.415	0.002	0.026	0.524	0.009	0.008	0.284	0	0.007	0.292
	N	47	47	47	47	47	47	47	47	47	47	47	47	47	47
2. HDJ	r_s		1	-0.04	**.557****	-0.035	**.574****	**.932****	**.543****	**.778****	0.169	**.476****	**-.360***	**.872****	**.370***
	p			0.789	0	0.817	0	0	0	0	0.256	0.001	0.013	0	0.011
	N		47	47	47	47	47	47	47	47	47	47	47	47	47
3. Mean daily precipitation (week b/h)	r_s			1	-0.285	.990**	-0.283	-0.26	-0.26	-.554**	-0.022	-0.085	0.241	-.440**	-0.204
	p				0.052	0	0.054	0.077	0.078	0	0.882	0.568	0.103	0.002	0.17
	N			47	47	47	47	47	47	47	47	47	47	47	47
4. Mean daily precipitation (week a/h)	r_s				1	-0.27	.999**	.615**	0.25	.679**	-.374**	.468**	-.670**	.706**	-0.086
	p					0.066	0	0	0.091	0	0.01	0.001	0	0	0.566
	N				47	47	47	47	47	47	47	47	47	47	47
5. Total precipitation (week b/h)	r_s					1	-0.268	-0.266	-.305*	-.534**	-0.066	-0.039	0.235	-.427**	-0.263
	p						0.068	0.071	0.037	0	0.66	0.792	0.112	0.003	0.074
	N					47	47	47	47	47	47	47	47	47	47
6. Total precipitation (week a/h)	r_s						1	.631**	0.253	.688**	-.366*	.463**	-.673**	.717**	-0.081
	p							0	0.086	0	0.011	0.001	0	0	0.59
	N						47	47	47	47	47	47	47	47	47
7. Ave min temperature (week b/h)	r_s							1	.568**	.857**	0.206	.380**	-.325*	.946**	.441**
	p								0	0	0.164	0.008	0.026	0	0.002
	N							47	47	47	47	47	47	47	47
8. Ave min temperature (week a/h)	r_s								1	.486**	.619**	0.126	-0.053	.540**	.869**
	p									0.001	0	0.398	0.723	0	0
	N								47	47	47	47	47	47	47
9. Ave max temperature (week b/h)	r_s									1	0.038	.455**	-.480**	.971**	0.283
	p										0.801	0.001	0.001	0	0.054
	N									47	47	47	47	47	47
10. Ave max temperature (week a/h)	r_s										1	-0.174	.625**	0.08	.898**
	p											0.241	0	0.592	0
	N										47	47	47	47	47
11. Diurnal temperature range (max - min) (week b/h)	r_s											1	-0.179	.476**	-0.071
	p												0.229	0.001	0.633
	N											47	47	47	47
12. Diurnal temperature range (max - min) (week a/h)	r_s												1	-.444**	.338*
	p													0.002	0.02
	N												47	47	47
13. Mean temperature (week b/h)	r_s													1	.344*
	p														0.018
	N													47	47
14. Mean temperature (week a/h)	r_s														1
	p														
	N														47

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). *p* is the 2-tailed significance. Ave = average, b/h = before hatch, a/h = after hatch

Table B-6. Relative contribution of factors on nest-level reproductive responses included in univariate generalized linear models. Significant p-values indicated with bold text.

Response	Covariate/factor		Parameter Estimate	95% Confidence interval Low	High	*p*-value
Clutch size	Julian hatch date		-0.011	-0.029	0.007	0.236
	Air pollution	BTEX (µg/m^3)	-0.898	-1.542	-0.255	**0.008**
		Hexane (µg/m^3)	4.881	-5.659	15.421	0.352
	Location	DR	-0.416	-1.750	0.918	0.530
		HV	-1.468	-3.343	0.406	0.120
		HW	-0.720	-2.334	0.893	0.369
		IN	-1.026	-3.093	1.042	0.319
		MB	0.353	-3.004	3.711	0.832
		MG	-0.348	-1.645	0.950	0.588
		PE	-0.677	-2.343	0.989	0.414
		RP	-0.455	-1.762	0.852	0.483
		SB	-0.351	-1.631	0.930	0.581
		WH	[a]			
Egg mass	Julian hatch date		-0.001	-0.018	0.015	0.859
	Air pollution	BTEX (µg/m^3)	0.189	-0.382	0.761	0.504
		Hexane (µg/m^3)	3.910	-5.451	13.271	0.400
	Location	DR	-0.536	-1.721	0.649	0.363
		HV	-1.049	-2.714	0.616	0.208
		HW	-0.337	-1.770	1.096	0.635
		IN	-2.071	-3.907	-0.235	**0.028**
		MB	-2.546	-5.674	0.583	0.107
		MG	-1.659	-2.811	-0.506	**0.006**
		PE	-0.986	-2.465	0.494	0.184
		RP	-0.211	-1.371	0.950	0.713
		SB	-0.889	-2.026	0.248	0.121
		WH	[a]			
Hatch success	Julian hatch date		-0.333	-0.934	0.268	0.268
	Air pollution	BTEX (µg/m^3)	-4.475	-25.584	16.633	0.668
		Hexane (µg/m^3)	6.737	-339.027	352.501	0.969

	Location	DR	-33.028	-76.801	10.745	0.134
		HV	-11.421	-72.914	50.073	0.707
		HW	-82.091	-135.018	-29.165	**0.003**
		IN	-23.125	-90.947	44.697	0.492
		MB	-16.398	-126.547	93.752	0.763
		MG	-30.911	-73.473	11.650	0.149
		PE	-11.230	-65.885	43.424	0.678
		RP	-16.318	-59.193	26.558	0.444
		SB	-39.703	-81.703	2.296	0.063
		WH	[a]			
Nest success	Julian hatch date		-0.019	-0.702	0.664	0.955
	Air pollution	BTEX ($\mu g/m^3$)	-7.881	-31.867	16.104	0.508
		Hexane ($\mu g/m^3$)	-76.265	-469.147	316.616	0.695
	Location	DR	-29.040	-78.779	20.698	0.243
		HV	-14.367	-84.240	55.507	0.678
		HW	-85.128	-145.267	-24.989	**0.007**
		IN	-10.017	-87.081	67.048	0.793
		MB	7.968	-117.191	133.128	0.898
		MG	-33.089	-81.450	15.272	0.173
		PE	4.331	-57.771	66.433	0.888
		RP	-12.183	-60.901	36.535	0.614
		SB	-34.023	-81.746	13.699	0.156
		WH	[a]			

[a] This parameter is set to zero because it is redundant.

Table B-7. Relative contribution of factors on individual nestling biometric and biochemical responses included in GEE models. Significant p-values indicated with bold text.

Response	Covariate/factor		Parameter Estimate	95% Confidence interval Low	High	*p*-value
Day 9 body mass (g)	Year	2014	4.270	-1.798	10.339	0.168
		2015	[a]			
	Location	DR	-4.885	-11.166	1.396	0.127
		HV	-2.746	-14.501	9.010	0.647
		HW	-5.747	-14.214	2.720	0.183
		IN	1.004	-13.563	15.572	0.893
		MB	30.073	1.160	58.985	**0.041**
		MG	-7.577	-13.513	-1.641	**0.012**
		PE	-5.811	-19.142	7.520	0.393
		RP	-7.275	-13.913	-0.638	**0.032**
		SB	-13.029	-19.906	-6.151	**0.000**
		WH	[a]			
	Julian hatch date		-0.116	-0.263	0.030	0.120
	Air pollution	BTEX (µg/m3)	34.721	12.210	57.233	**0.003**
		Hexane (µg/m3)	94.763	-8.868	198.394	0.073
		BTEX * Hexane	-95.041	-150.690	-39.393	**0.001**
Day 9 wing chord (mm)	Year	2014	2.085	-4.906	9.076	0.559
		2015	[a]			
	Location	DR	-0.871	-8.949	7.207	0.833
		HV	-1.858	-15.025	11.308	0.782
		HW	-3.903	-11.838	4.032	0.335
		IN	1.398	-18.084	20.879	0.888
		MB	11.393	-26.169	48.954	0.552
		MG	-3.451	-7.847	0.945	0.124
		PE	-4.218	-19.454	11.018	0.587
		RP	2.844	-2.760	8.448	0.320
		SB	-6.044	-11.682	-0.406	**0.036**
		WH	[a]			
	Julian hatch date		-0.003	-0.145	0.139	0.966

	Air pollution	BTEX (µg/m3)		3.992	-15.489	23.474	0.688
		Hexane (µg/m3)		0.711	-93.559	94.980	0.988
		BTEX Hexane	*	-11.846	-58.811	35.118	0.621
Day 9 tarsal length (mm)	Year	2014		0.340	-0.578	1.258	0.468
		2015		[a]			
	Location	DR		-0.458	-1.374	0.459	0.328
		HV		-1.117	-2.728	0.493	0.174
		HW		-0.728	-1.657	0.201	0.124
		IN		-0.655	-3.021	1.712	0.588
		MB		0.482	-4.072	5.036	0.836
		MG		-1.021	-1.814	-0.228	**0.012**
		PE		-1.423	-3.375	0.529	0.153
		RP		-0.783	-1.375	-0.191	**0.010**
		SB		-1.245	-1.967	-0.522	**0.001**
		WH		[a]			
	Julian hatch date			-0.018	-0.033	-0.003	**0.021**
	Air pollution	BTEX (µg/m3)		2.080	-1.504	5.664	0.255
		Hexane (µg/m3)		8.249	-5.344	21.842	0.234
		BTEX Hexane	*	-5.581	-13.671	2.509	0.176
Day 9 scaled mass index	Year	2014		5.855	0.664	11.045	**0.027**
		2015		[a]			
	Location	DR		-3.075	-8.508	2.357	0.267
		HV		6.492	-7.159	20.143	0.351
		HW		-0.784	-7.878	6.310	0.829
		IN		5.494	-9.295	20.283	0.467
		MB		37.429	4.946	69.913	**0.024**
		MG		-0.839	-3.823	2.145	0.582
		PE		2.828	-9.114	14.771	0.643
		RP		-4.684	-9.821	0.453	0.074
		SB		-7.329	-11.892	-2.765	**0.002**
		WH		[a]			
	Julian hatch date			0.015	-0.125	0.155	0.834
	Air pollution	BTEX (µg/m3)		36.887	22.457	51.317	**0.000**

		Hexane (µg/m3)		72.175	-8.907	153.256	0.081
		BTEX Hexane	*	-97.027	-134.166	-59.887	**0.000**
PHA response	Year	2014		-0.211	-0.453	0.032	0.089
		2015		[a]			
	Location	DR		0.116	-0.120	0.352	0.336
		HV		-0.035	-0.659	0.589	0.913
		HW		-0.310	-0.601	-0.020	**0.036**
		IN		0.378	-0.368	1.124	0.321
		MB		-0.219	-1.848	1.409	0.792
		MG		0.193	0.052	0.334	**0.007**
		PE		0.353	-0.248	0.953	0.250
		RP		0.175	-0.061	0.410	0.147
		SB		0.140	-0.109	0.389	0.270
		WH		[a]			
	Julian hatch date			0.002	-0.003	0.008	0.368
	Air pollution	BTEX (µg/m3)		-0.526	-1.253	0.200	0.156
		Hexane (µg/m3)		-0.966	-4.532	2.601	0.596
		BTEX Hexane	*	0.991	-0.862	2.844	0.295
Day 15 body mass (g)	Year	2014		4.225	-3.827	12.277	0.304
		2015		[a]			
	Location	DR		-10.070	-17.859	-2.281	**0.011**
		HV		-15.492	-28.899	-2.085	**0.024**
		HW		2.486	-6.631	11.603	0.593
		IN		-24.813	-47.008	-2.618	**0.028**
		MB		-31.947	-77.049	13.155	0.165
		MG		-3.159	-8.976	2.657	0.287
		PE		-25.550	-45.144	-5.956	**0.011**
		RP		-11.597	-16.861	-6.333	**0.000**
		SB		-13.486	-21.287	-5.686	**0.001**
		WH		[a]			
	Julian hatch date			-0.179	-0.316	-0.042	**0.010**
	Air pollution	BTEX (µg/m3)		15.028	-14.550	44.605	0.319
		Hexane (µg/m3)		87.287	-47.950	222.523	0.206

		BTEX * Hexane	-18.518	-98.867	61.831	0.651
Day 15 tarsal length (mm)	Year	2014	0.783	0.202	1.363	**0.008**
		2015	[a]			
	Location	DR	-1.060	-1.709	-0.411	**0.001**
		HV	-0.590	-1.594	0.414	0.249
		HW	-0.745	-1.614	0.125	0.093
		IN	-1.299	-2.697	0.099	0.069
		MB	-0.677	-3.110	1.756	0.586
		MG	-0.944	-1.501	-0.388	**0.001**
		PE	-1.510	-2.611	-0.408	**0.007**
		RP	-1.451	-2.007	-0.895	**0.000**
		SB	-1.494	-2.202	-0.786	**0.000**
		WH	[a]			
	Julian hatch date		-0.027	-0.039	-0.014	**0.000**
	Air pollution	BTEX (µg/m3)	2.509	0.439	4.579	**0.017**
		Hexane (µg/m3)	3.230	-6.137	12.597	0.499
		BTEX * Hexane	-4.977	-9.753	-0.201	**0.041**
Day 15 wing chord (mm)						
1) location	Location	DR	-1.746	-3.381	-0.110	**0.036**
		HV	-2.200	-3.215	-1.186	**0.000**
		IN	-0.179	-5.720	5.363	0.950
		MG	1.403	-0.911	3.717	0.235
		PE	-2.544	-6.549	1.461	0.213
		RP	-1.330	-5.422	2.761	0.524
		SB	-5.507	-6.089	-4.924	**0.000**
		WH	[a]			
	Julian hatch date		0.079	-0.038	0.195	0.186
2) air pollution	Julian hatch date		0.059	-0.087	0.204	0.430
	Air pollution	BTEX (µg/m3)	-1.131	-41.190	38.928	0.956
		Hexane (µg/m3)	-9.078	-223.027	204.872	0.934
		BTEX * Hexane	2.854	-122.849	128.556	0.965

Day 15 scaled mass index	Year	2014	-9.855	-16.754	-2.957	**0.005**
		2015	[a]			
	Location	DR	2.849	-3.078	8.777	0.346
		HV	-8.439	-17.810	0.932	0.078
		HW	13.025	5.624	20.426	**0.001**
		IN	-11.989	-28.572	4.593	0.156
		MB	-23.920	-56.610	8.770	0.152
		MG	7.912	4.300	11.524	**0.000**
		PE	-10.394	-24.292	3.503	0.143
		RP	5.609	0.450	10.767	**0.033**
		SB	3.515	-3.261	10.292	0.309
		WH	[a]			
	Julian hatch date		0.140	-0.017	0.297	0.080
	Air pollution	BTEX (µg/m3)	-8.816	-30.623	12.992	0.428
		Hexane (µg/m3)	64.398	-33.424	162.220	0.197
		BTEX * Hexane	29.875	-27.012	86.763	0.303
SMI change (day 9-15)	Year	2014	-14.807	-23.569	-6.045	**0.001**
		2015	[a]			
	Location	DR	5.294	-2.588	13.177	0.188
		HV	-13.518	-28.271	1.235	0.073
		HW	13.775	3.013	24.538	**0.012**
		IN	-16.945	-38.567	4.677	0.125
		MB	-58.552	-102.512	-14.593	**0.009**
		MG	7.765	3.409	12.120	**0.000**
		PE	-13.869	-29.524	1.785	0.082
		RP	9.319	1.231	17.407	**0.024**
		SB	9.254	1.771	16.736	**0.015**
		WH	[a]			
	Julian hatch date		0.150	-0.080	0.380	0.202
	Air pollution	BTEX (µg/m3)	-41.562	-65.815	-17.308	**0.001**
		Hexane (µg/m3)	-5.214	-132.889	122.461	0.936
		BTEX * Hexane	117.507	52.669	182.346	**0.000**
	Year	2014	-0.028	-0.073	0.018	0.231

Scaled spleen mass index		2015	[a]			
	Location	DR	0.040	0.001	0.078	**0.046**
		HV	-0.035	-0.111	0.041	0.364
		HW	-0.012	-0.071	0.047	0.686
		IN	-0.038	-0.114	0.038	0.330
		MB	-0.142	-0.275	-0.008	0.038
		MG	0.036	-0.013	0.085	0.149
		PE	-0.011	-0.082	0.061	0.773
		RP	0.055	0.004	0.107	**0.035**
		SB	0.022	-0.031	0.075	0.415
		WH	[a]			
	Julian hatch date		0.001	0.000	0.002	0.098
	Air pollution	BTEX (µg/m3)	-0.120	-0.313	0.074	0.226
		Hexane (µg/m3)	-0.043	-0.924	0.839	0.924
		BTEX * Hexane	0.329	-0.163	0.821	0.190
Scaled bursal mass index	Year	2014	-0.002	-0.048	0.043	0.917
		2015	[a]			
	Location	DR	-0.025	-0.059	0.009	0.157
		HV	0.074	0.010	0.138	**0.022**
		HW	-0.013	-0.066	0.039	0.624
		IN	0.061	-0.022	0.143	0.150
		MB	0.239	0.104	0.374	**0.001**
		MG	-0.011	-0.037	0.014	0.391
		PE	0.009	-0.055	0.074	0.778
		RP	-0.025	-0.067	0.017	0.238
		SB	-0.036	-0.100	0.029	0.282
		WH	[a]			
	Julian hatch date		0.000	-0.001	0.001	0.682
	Air pollution	BTEX (µg/m3)	0.090	-0.055	0.235	0.223
		Hexane (µg/m3)	-0.279	-0.993	0.435	0.444
		BTEX * Hexane	-0.255	-0.605	0.094	0.152
Scaled liver mass index	Year	2014	-0.951	-1.768	-0.133	**0.023**
		2015	[a]			

	Location	DR	0.572	-0.024	1.169	0.060
		HV	1.099	-0.142	2.340	0.083
		HW	0.698	-0.237	1.634	0.144
		IN	1.372	-0.066	2.809	0.061
		MB	2.471	-1.033	5.974	0.167
		MG	0.946	0.354	1.538	**0.002**
		PE	0.967	-0.181	2.116	0.099
		RP	1.326	0.583	2.068	**0.000**
		SB	1.155	0.185	2.125	**0.020**
		WH	[a]			
	Julian hatch date		0.023	0.002	0.044	**0.031**
	Air pollution	BTEX (µg/m3)	-1.999	-5.222	1.225	0.224
		Hexane (µg/m3)	-7.789	-20.079	4.500	0.214
		BTEX * Hexane	4.323	-3.966	12.612	0.307
EROD (2014)						
1) location	Location	DR	59.843	11.841	107.845	**0.015**
		HV	13.720	0.758	26.682	**0.038**
		HW	44.048	18.659	69.437	**0.001**
		IN	52.921	42.755	63.088	**0.000**
		MB	29.406	2.566	56.247	**0.032**
		MG	43.803	30.043	57.562	**0.000**
		PE	50.104	22.649	77.560	**0.000**
		RP	47.753	23.221	72.284	**0.000**
		SB	88.100	38.707	137.492	**0.000**
		WH	[a]			
	Julian hatch date		-0.490	-1.236	0.257	0.199
2) air pollution	Julian hatch date		-1.460	-2.145	-0.774	**0.000**
	Air pollution	BTEX (µg/m3)	92.000	48.392	135.609	**0.000**
		Hexane (µg/m3)	-148.350	-341.200	44.499	0.132
		BTEX * Hexane	-118.375	-211.754	-24.997	**0.013**
EROD (2015)						
1) location	Location	DR	192.369	182.359	202.379	**0.000**

		HV	71.356	-68.935	211.647	0.319
		IN	362.982	167.642	558.322	**0.000**
		MG	276.378	234.625	318.131	**0.000**
		PE	146.539	121.415	171.662	**0.000**
		RP	235.518	161.055	309.981	**0.000**
		SB	125.841	109.158	142.525	**0.000**
		WH	[a]			
	Julian hatch date		-1.209	-4.545	2.128	0.478
2) air pollution	Julian hatch date		1.436	-2.459	5.331	0.470
	Air pollution	BTEX (µg/m3)	-882.709	-2257.633	492.216	0.208
		Hexane (µg/m3)	-6853.415	-14255.807	548.978	0.070
		BTEX * Hexane	3184.713	-1066.016	7435.442	0.142
TBARS	Year	2014	12.997	3.463	22.531	**0.008**
		2015	[a]			
	Location	DR	-13.378	-23.080	-3.677	**0.007**
		HV	2.926	-17.378	23.230	0.778
		HW	-25.315	-36.738	-13.892	**0.000**
		IN	3.810	-25.665	33.286	0.800
		MB	52.434	-17.620	122.488	0.142
		MG	-12.000	-21.358	-2.643	**0.012**
		PE	-2.700	-25.937	20.536	0.820
		RP	-23.504	-31.213	-15.794	**0.000**
		SB	-22.770	-31.562	-13.977	**0.000**
		WH	[a]			
	Julian hatch date		-0.415	-0.609	-0.222	**0.000**
	Air pollution	BTEX (µg/m3)	36.095	-7.123	79.314	0.102
		Hexane (µg/m3)	18.300	-161.125	197.726	0.842
		BTEX * Hexane	-106.776	-230.613	17.060	0.091
tGSH	Year	2014	-3.344	-4.142	-2.546	**0.000**
		2015	[a]			
	Location	DR	0.059	-0.555	0.673	0.851
		HV	1.853	0.183	3.523	**0.030**
		HW	-0.118	-1.289	1.053	0.843

		IN	4.156	2.077	6.234	**0.000**
		MB	2.719	-1.158	6.596	0.169
		MG	1.893	1.355	2.431	**0.000**
		PE	2.416	0.999	3.832	**0.001**
		RP	0.191	-0.317	0.699	0.461
		SB	1.145	-0.091	2.381	0.069
		WH	[a]			
	Julian hatch date		0.008	-0.009	0.025	0.364
	Air pollution	BTEX (µg/m3)	-6.935	-10.607	-3.263	**0.000**
		Hexane (µg/m3)	-34.209	-53.101	-15.317	**0.000**
		BTEX * Hexane	16.588	6.686	26.490	**0.001**
rGSH:GSSG (2015)						
1) location	Location	DR	-1.647	-4.331	1.038	0.229
		HV	-9.243	-14.532	-3.954	**0.001**
		IN	12.028	0.082	23.974	**0.048**
		MG	-8.833	-18.585	0.918	0.076
		PE	2.733	-1.815	7.281	0.239
		RP	-0.965	-43.682	41.752	0.965
		SB	-3.924	-8.398	0.549	0.086
		WH	[a]			
	Julian hatch date		0.776	-0.119	1.671	0.089
2) air pollution	Julian hatch date		-0.228	-0.616	0.160	0.250
	Air pollution	BTEX (µg/m3)	46.358	-32.893	125.609	0.252
		Hexane (µg/m3)	215.578	-183.533	614.689	0.290
		BTEX * Hexane	-137.853	-383.878	108.172	0.272

[a] This parameter is set to zero because it is redundant.

FIGURES

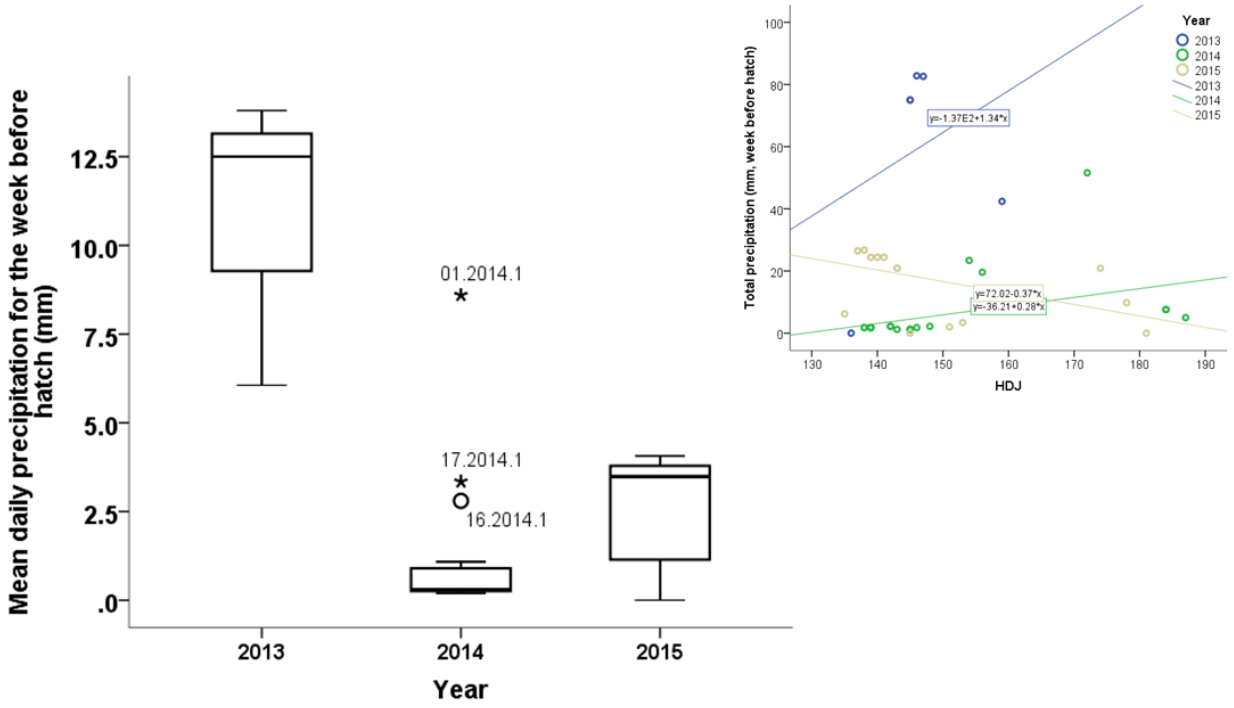

Figure B-7. The precipitation (rain + snow) in Calgary for May–June of 2013, 2014 and 2015 (left), demonstrating the dramatic effect of year. Inset: when annual precipitation (same months) is graphed by hatch date, it is clear that HDJ and precipitation show consistent relationship.

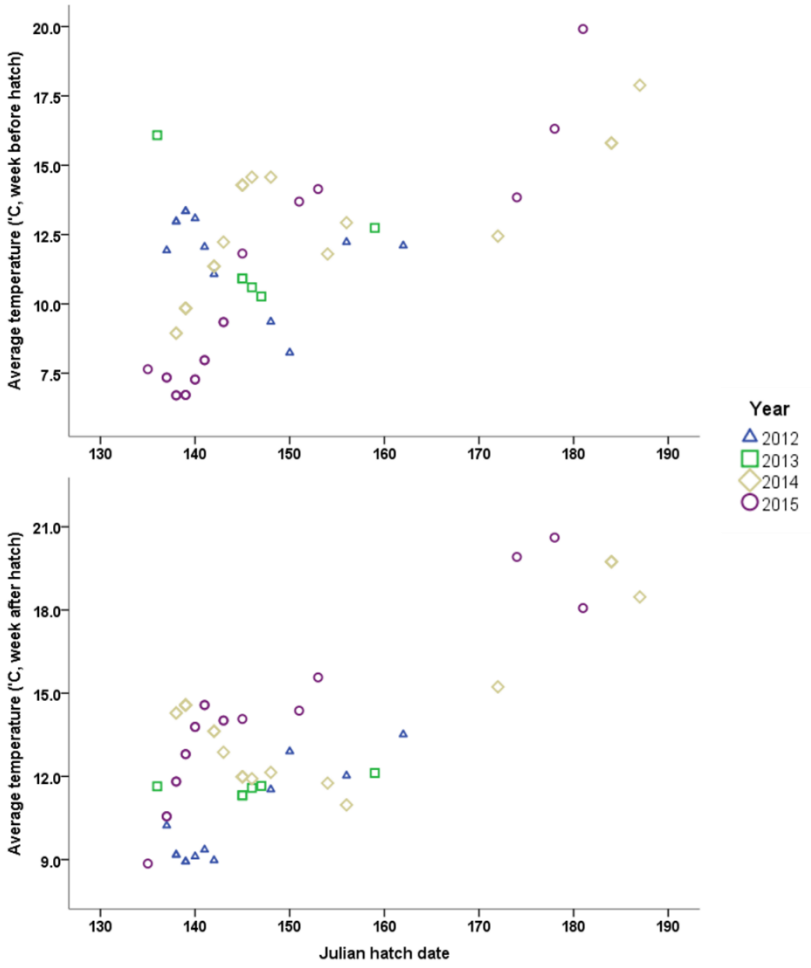

Figure B-8. Linear relationship between average daily temperature and hatch date, for the week before (above) and after (below) hatch.

References

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2. Nilsen, B. M.; Berg, K.; A., G., Induction of cytochrome P450 1A (CYP1A) in fish: a biomarker for environmental pollution. *Methods in Molecular Biology* **1998,** *107*, 423-38.
3. Rodríguez-Estival, J.; North, M. A.; Smits, J. E. G., Sublethal health effects in laboratory rodents from environmentally relevant exposures to oil sands contaminants. *Environmental Toxicology and Chemistry* **2015,** *34* (12), 2884-2897.

APPENDIX C: EUROPEAN STARLINGS IN CALGARY, ALBERTA

C.1. Introduction

European starlings (*Sturnus vulgaris*) are a successful, cavity-nesting songbird (passerine) found near human settlements in Europe, with invasive populations introduced to North America and parts of Australia and South Africa (Figure C-1).

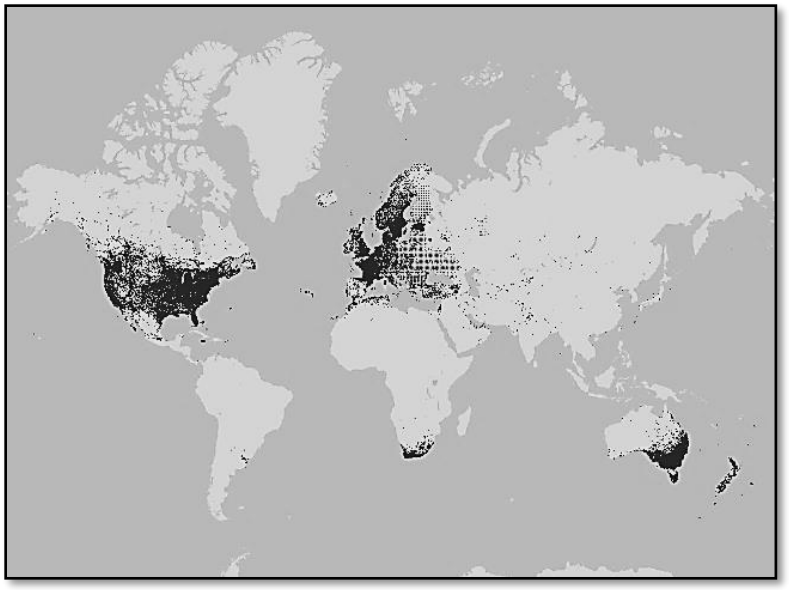

Figure C-9. Map of *Sturnus vulgaris* distribution in 2010. Available under the Open Database License from http://www.gbif.org/species/2489105, © OpenStreetMap contributors.

Starlings have been the focus of extensive research describing starling life history and impact as an invasive species, breeding biology, foraging decisions and diet. Adapting well to captivity, starlings have been used as a model experimental animal in a wide range of fields, from neuro-endocrinology, behaviour and vision, to mechanics of flight, parasitology and stress. A Web of Science literature search produced 595 results, published between 1930 and 2015 (Box C-1).

Box C-1. Search terms used in Web of Science literature review (2016/01/26):

TI=("common starling" OR "european starling" AND bird) OR TI=("sturnus vulgaris") NOT AU=(starling) Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Timespan=All years

European starlings were chosen as the sentinel species for this thesis project, a study investigating the effects of air pollution on wild birds in the city of Calgary, Alberta, Canada. Starlings are an ideal sentinel since they are an invasive species with large, successful populations globally and in the study area, they adapt readily to artificial nest boxes, and are largely unaffected by investigator presence (Crossner, 1977). Beyond these factors, there is a wealth of information available on the life history of European starlings in North America (Kessel, 1957, Collins and De Vos, 1966).

There have been numerous studies investigating the effects of different environmental toxicants on starlings, for example, pesticides (Rattner and Grue, 1990, Yusufu, 1996, Wolfe and Kendall, 1998, Parker and Goldstein, 2000, Flahr *et al.*, 2015), heavy metals (Congiu *et al.*, 2000, Carlson *et al.*, 2014), industrial toxicants (Zahara *et al.*, 2015), and wastewater contaminated with endocrine-disrupting pharmaceuticals (Markman *et al.*, 2011). Studies using wild starlings as sentinels of environmental contamination are less common, yet examples exist for pesticides, metals, flame retardants, and industrial toxicants (Grue *et al.*, 1986, Akins *et al.*, 1993, Arenal *et al.*, 2004, Erratico *et al.*, 2015), including the Western Beef Productivity Study, a large, multifaceted study investigating the effects of oil and natural gas emissions on cattle and wildlife (wild bird) health in the Canadian prairies (WISSA, 2006).

Before we can evaluate whether exposure to air pollution influences starling reproduction, and the growth, development and survival of nestling starlings, it is important to determine what normal values are for this population. Therefore, this chapter describes reproductive data for this population, compares it to that of other populations, and describes some local biological and non-toxicological, anthropogenic factors affecting starling health and fitness.

C.2. Materials and Methods

C.2.1. Nest boxes, distribution and description of locations

All nest boxes were placed approximately 3 m above the ground in deciduous trees [predominantly trembling aspen (*Populus tremuloides*), or balsam poplar (*Populus balsamifera*)] or utility poles in urban parks and natural areas in the city of Calgary (Figure C-2 and C-3). Nest box holes were reinforced with 3 mm aluminium plate to prevent Eastern grey squirrels (*Sciurus carolinensis*) from enlarging the holes to gain access to the nests. The boxes were usually oriented on the leeward side of the tree to reduce nest disturbance from wind and rain.

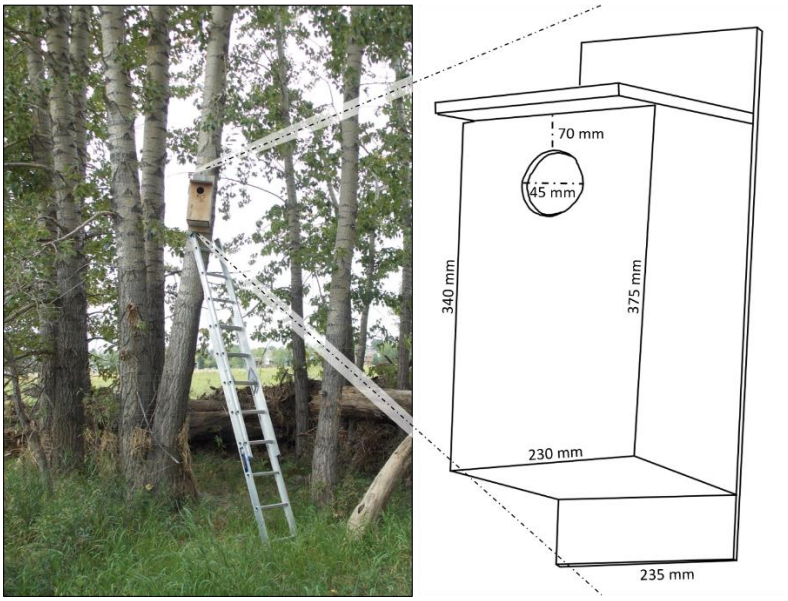

Figure C-10. Nest box dimensions and attachment to trees

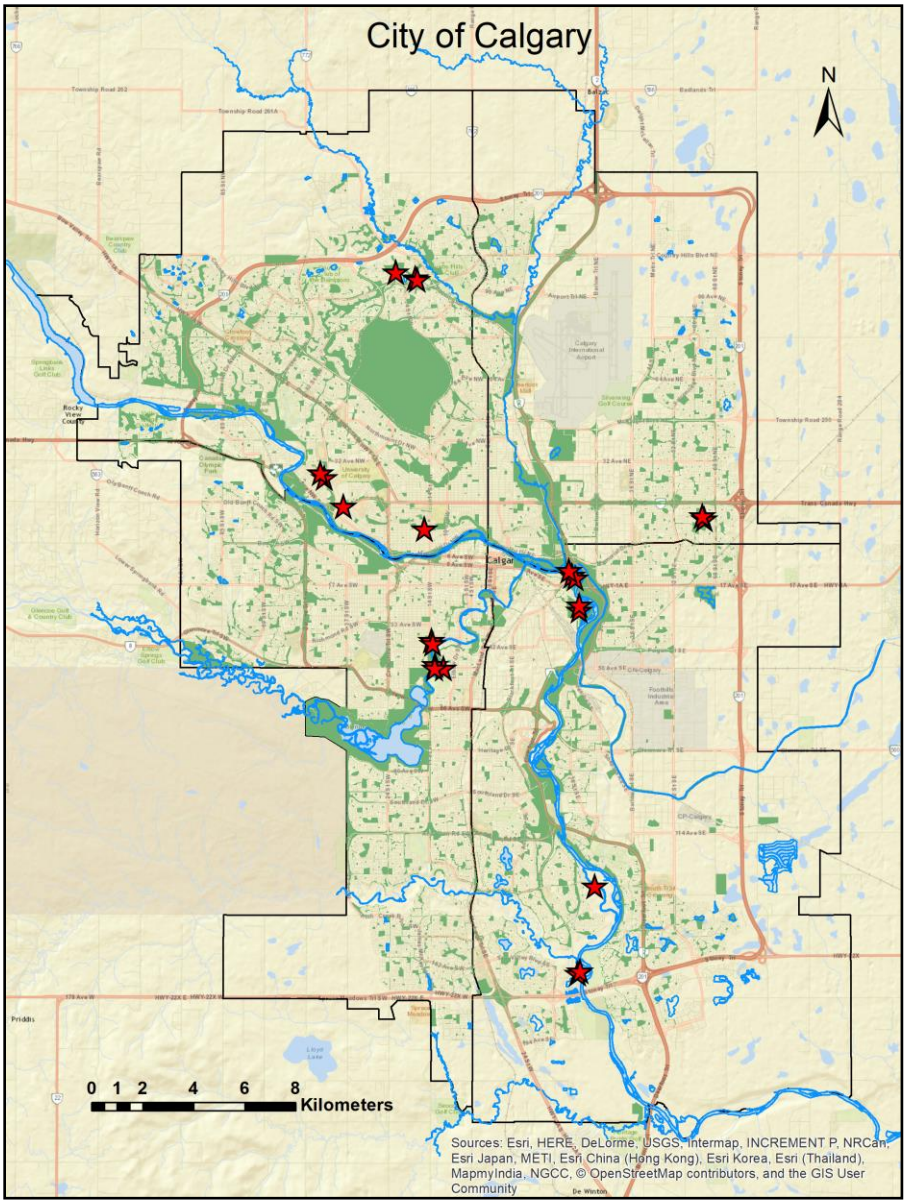

Figure C-11. Map of the city of Calgary showing the distribution of nest sites (red stars), in relation to natural areas (green), hydrology (blue), and underlying road network (brown) and city quadrants (black).

The parks were predominately used for dog-walking, as picnic sites or for other outdoor activities, and largely consisted of grassland (groomed or natural) interspersed with patches of deciduous and coniferous trees (Figure C-4). Some of the parks were closer to urban infrastructure (foot- and bicycle pathways, suburban housing, fuel stations & roads), others were beside the two major

rivers (i.e., the Bow or Elbow Rivers) and their streams with some riparian habitat, while most boxes were on grasslands and at higher elevation.

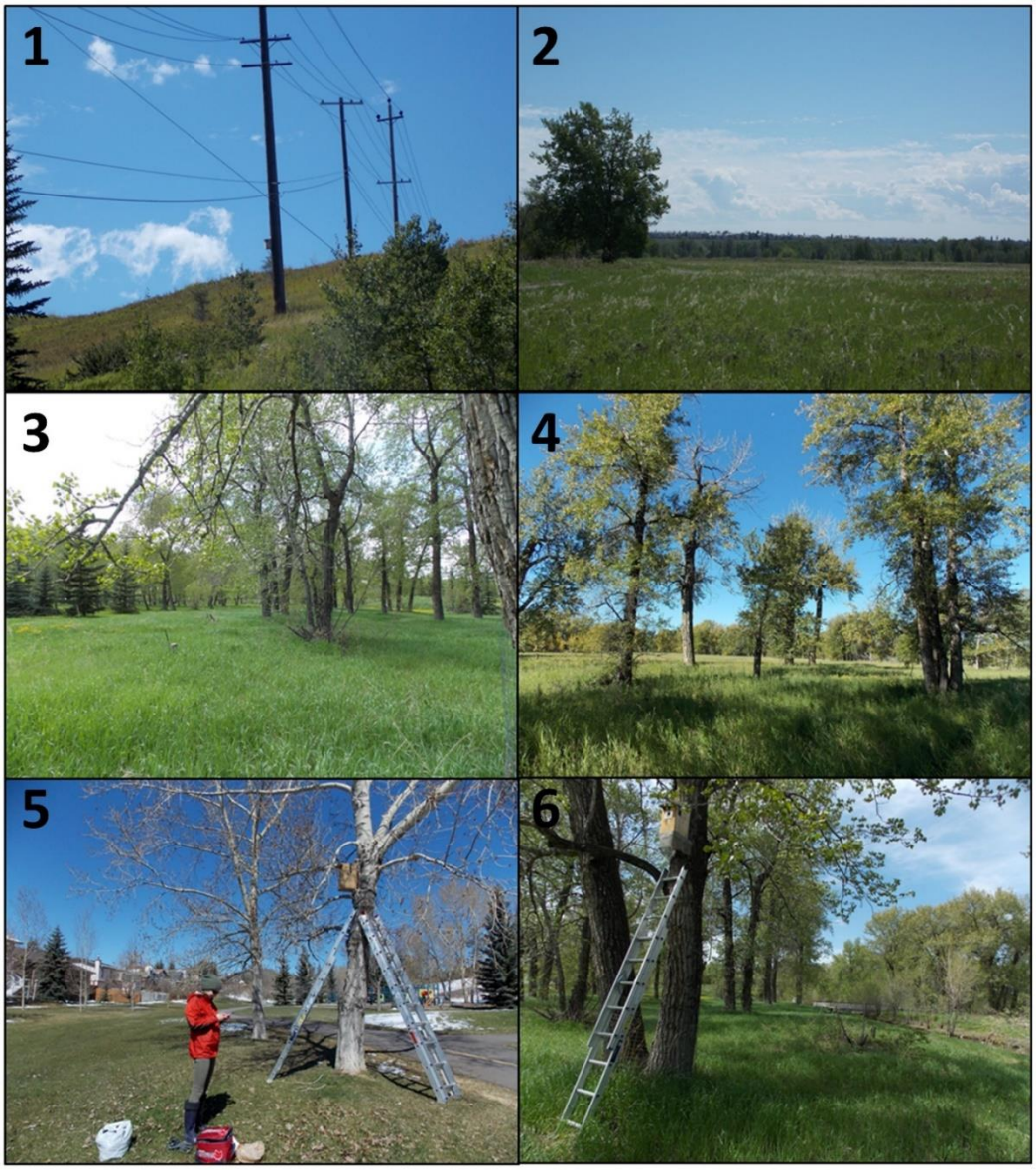

Figure C-12. Examples of nest box locations in natural (1-4, 6) and groomed (5) urban parks. Note the long, uncut grass in the former and the bicycle path, houses and mown lawn in the latter. 1) Sandy Beach, 2) Deer Run, 3) Pearce Estate, 4) Hull's Wood, 5) Edgemont (like Hidden Valley), 6) Pearce Estate.

The possible biological effect of different landscape types was investigated by categorizing the parks as natural or groomed (Table C-1) depending on whether the predominate feeding areas of the birds was likely groomed lawn or natural scrub grassland. All the parks had similar tree composition and human presence (or disturbance), with predominant use by the public either in transit along the pathways or for outdoor leisure.

Table C-8. Examples of the natural and groomed study sites in Calgary

Site	Abbreviation	Park type	Riparian zones
Fish Creek Provincial Park			
Deer Run	DR	Natural	Yes
Hulls Wood	HW	Natural	Yes
Pearce Estate	PE	Natural	Yes
Inglewood	IN	Natural	Yes
River Park	RP	Natural	Yes
Hidden Valley	HV	Groomed	No
Montgomery	MG	Groomed	No
Marlborough	MB	Groomed	No
Woods Homes	WH	Groomed	No
Sandy Beach	SB	Groomed	Yes

C.2.2. Population monitoring and measurements

Nest boxes were cleaned in August–September in preparation for the next year's breeding season, to decrease the likelihood of nest parasites surviving overwinter. The boxes were checked every 2–3 days from the first week of May for signs of nesting activity (including nest and egg cup building) until at least one egg was found. The nest was re-checked approximately one week later, the total number of eggs counted and weighed to the nearest 0.5 g (100 g Pesola® micro spring scale), and the onset of incubation calculated (date penultimate egg was laid, assuming one egg laid per day). Hatch date was predicted (date of penultimate egg + 13 days) and the nest checked daily from before the predicted date, until all eggs had hatched.

The number of nestlings was counted (hatching success = # nestlings/# eggs laid), and then the nest was left undisturbed until nine days after hatch. Nestlings were individually identified using temporary coloured leg bands, weighed (±1 g, 300 g Pesola® spring scale), and the tarsal and wing chord lengths were measured using a digital caliper (±0.1 mm) and modified ruler (±1 mm), respectively (Figure C-5). The nestlings were revisited at fifteen days after hatching, weighed and measured again. Nest success (# fledglings/# eggs laid) and nest mortality (#hatched - #fledged) were determined for each nest.

Figure C-13. Morphometric measurements for nestling starlings

Nestling condition was assessed using the scaled mass index (SMI), a method of normalizing mass for differences in skeletal size validated for many species, including starlings (Peig and Green, 2009). Nestling SMI was calculated as described in detail in North *et al.* (2017). Day 9 and 15 scaled mass indices were normalized for the respective day 9 and 15 tarsal lengths, 29.32 mm and 30.10 mm, respectively.

All procedures performed in this study were approved by the University of Calgary Animal Care Committee, in accordance with Animal Use Protocols SHC11R-15 and AC15-0070.

C.2.3. Diet

As part of an undergraduate, summer student subproject in 2015 (C. Lengkeek, unpublished), nestling diets were assessed by examining the stomach contents of 2–3 birds per nest (sacrificed as part of the overarching toxicological study). Stomach contents were removed and weighed (±0.01 g), and stored in 70% ethanol until they could be examined. Contents were dried under nitrogen, and separated into plant matter (mostly grass), insect or insect parts, earthworms or caterpillars, and berries or fruit. The relative proportion of each dietary component was calculated and expressed as a percentage. The different dietary sources were evaluated by hatch date and park type, as well as nestling growth and body condition.

C.2.4. Hatch date, season and weather

Hatch date Julian (HDJ, days numbered consecutively from 1 January) was assigned as the date on which most of the eggs from each nest had hatched. Clutches were clustered into two main groups, those hatching in late May–June, and those hatching in mid–late July; the breeding season was thus divided into 'early' (HDJ < 170, or 18–19 June) and 'late' broods (everything hatching thereafter). The early and late broods may have been first and second broods by the same parents, but this study was not designed to identify parents.

The effect of weather on reproduction and nestling growth and development in Calgary was investigated using daily weather data for April to July of 2013–2015 measured at the Calgary International Airport and made available to the public (Environment Canada, 2013–2015). Total and daily precipitation, minimum, maximum and average temperatures and the daily diurnal temperature range were extracted for the week before and after the HDJ for each nest.

C.2.5. Statistical analysis

Spearman correlation was used to identify relationships between biological responses and the possible linear abiotic predictors, and the strength of association, r_S, and significance, *p*-value, are reported where significant. The relative contribution of year, location and hatch date on responses

(e.g., clutch size and egg mass) were calculated using generalized linear regression, with year and location included as factors, and hatch date as a covariate, using a Poisson loglinear model for count-variables. The effect of park type (i.e., riverine or upland, and natural or groomed) on body condition was analyzed using generalized linear model, with river access, and brood size (factors), with pairwise comparisons. HDJ and landscape around nest were not significant and not included in models. The variation in precipitation among years was assessed using Multivariate Analysis of Variance, (year as fixed factor, hatch date as covariate, precipitation before and after hatch as dependent variables) with Bonferroni correction for multiple comparisons. Non-parametric, Independent Samples Median Test was used to evaluate the difference in diet (as stomach contents) between riverine and non-riverine areas, and between groomed and natural areas. All analyses were done using SPSS statistical software, version 24.0 (IBM SPSS Inc., Chicago, IL, USA), two-tailed significance set to $p<0.05$.

C.3. Results and Discussion

Of 31 nest boxes deployed in 2012, two boxes were occupied by squirrels, one by kestrels, and one by flickers; two starling broods died, one by predation (presumably by squirrels). In 2013, of 35 nest boxes, one brood of starlings failed to hatch, seven boxes were occupied by squirrels and two by flickers. Of 44 boxes monitored in 2014, one nest with eggs was abandoned, and seven with constructed nest cups were not used. Twenty-five boxes were monitored in 2015; four nests were built but abandoned, three broods died within the first week after hatch, and one box was occupied by tree swallows.

Sixty-one clutches were studied in total: 12 in 2012, 6 in 2013, 24 in 2014 and 19 in 2015. Habituation of naïve starling populations to the nest boxes (2012), inclement weather leading to hatching failure and nest abandonment (2013), expanded monitoring to new sites and squirrel-proofing of boxes (2014), and streamlining of the monitored sites for logistical reasons (2015), explain some variation in sample size among years.

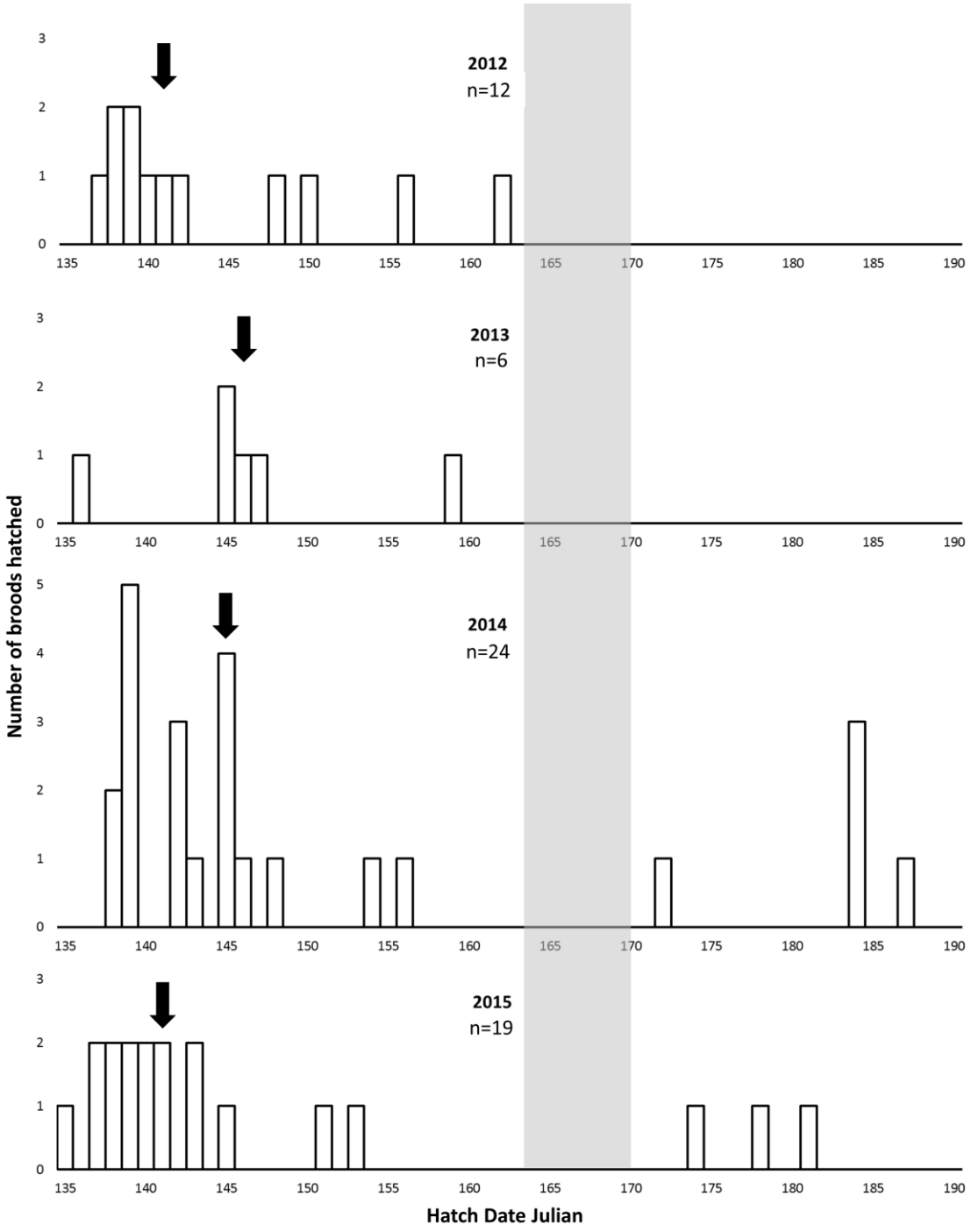

Figure C-14. Hatch date distribution during 2012–2015. The grey box indicates the approximate cut-off for early- (left) versus late-broods (right). Black arrows represent the median hatch dates for each year.

Timing of breeding varied very little from year to year (Figure C-6), with the earliest nests (of those monitored) hatching on HDJ 137, 136, 138 or 135, for 2012, 2013, 2014 and 2015, respectively (15–18 May), corresponding with findings in previous studies (Ricklefs and Peters, 1979, Pinxten *et al.*, 1990). While breeding extends later in the season for some years (Figure 3-6), median hatch dates were also consistent, varying from 141–146 (20–26 May). There were 53 early clutches and 8 late clutches spanning these years. The absence of second- or late broods in 2012 and 2013 was because monitoring was stopped in July in those years.

Hatch date was significantly correlated with the minimum temperatures before and after hatch (r_S=0.713 and 0.630, respectively, p<0.0001, n=61), implying that more eggs hatch as the season progresses from spring to summer. Kessel (1957) and Collins and De Vos (1966), from studies of starling populations in Ithaca, New York and Guelph, Ontario, respectively, suggest that in addition to increasing photoperiod, average temperatures need to exceed 40˚F (4.4˚C) for at least 11–17 days before laying to stimulate rapid gonadal development, a common hypothesis for seasonal-breeders (Williams *et al.*, 2015). This doesn't appear to be the case in this population (Figure C-7), which may support a more recent theory that mid-winter temperatures predict laying date indirectly via the temperature-dependent development of starlings' insect prey (Williams *et al.*, 2015); however, we did not check the effect of mid-winter temperatures since it was beyond the scope of this study.

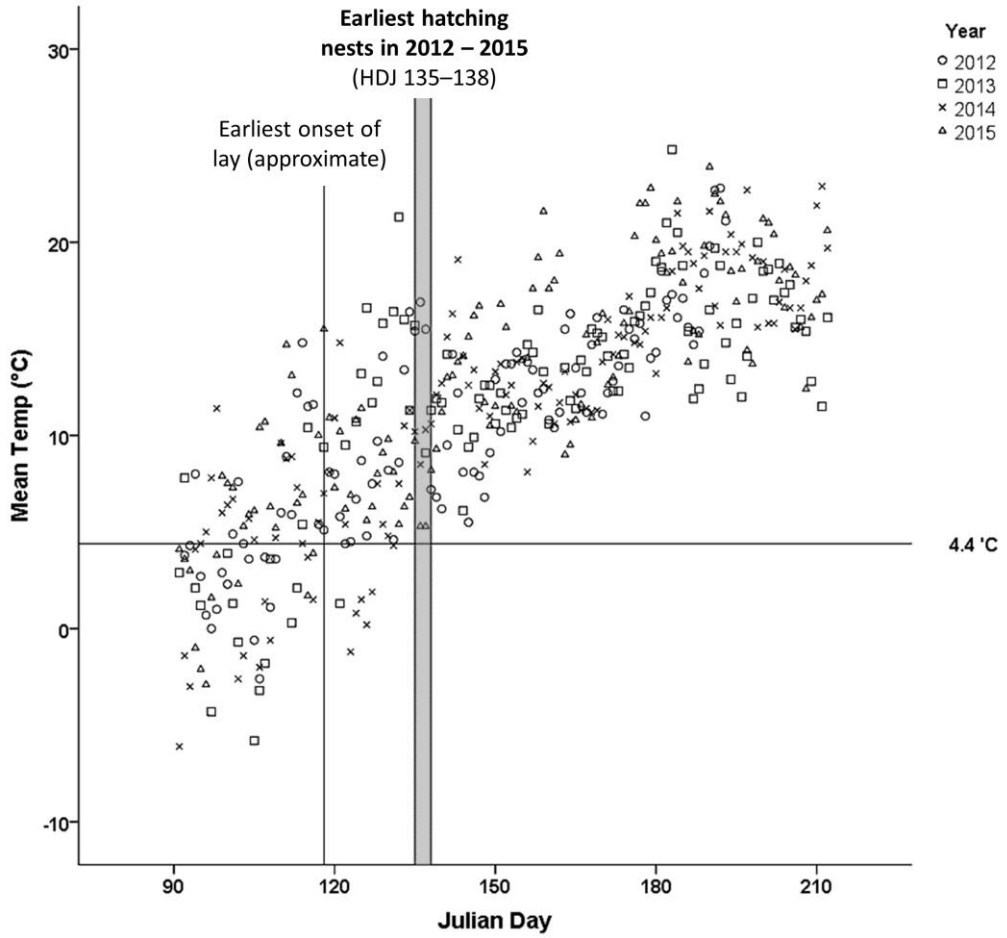

Figure C-15. Average temperatures for the 2012 to 2015 nesting seasons, with the earliest approximate onset of lay (vertical line) and the range of earliest hatching dates (shaded) for 2012-2015.

Clutch size varied from 3–7, with an average of 5 of eggs per nest (4.9 ± 0.1, mean ± standard error of the mean, SEM). Hatching success and nestling survival were on par with other populations (Table C-2), with only eight nests losing one or more chicks after hatching.

Table C-9. European starling reproduction: comparison of results between studies. Mean ± standard error of the mean (SEM) is reported except where specified otherwise.

	Clutch	Smith and Bruun (1998)	Collins and De Vos (1966) [a]	Ricklefs and Peters (1979)	This study [a]
Country		Sweden	ON, Canada	PA, USA	AB, Canada
Main land-use		Agriculture	Mixed agriculture: pasture, field & forest	Woods, pasture, crops	Urban parks, deciduous & spruce trees
Egg mass (g)	I	5.9–8.4 [b]			6.47 ± 0.12
	II				6.33 ± 0.27
Clutch size	I	4–5 [b]	5.6 ± 0.05	4.7	5.1 ± 0.1
	II		5.0 ± 0.06	4.1	4.1 ± 0.3
Hatch success (%) [c]	I		92	86	83
	II		83	72	88
Fledgling survival (%) [c]	I	73	94	70	100
	II		89	43	
Nest success (%) [c]	I		83		80
	II		71		88

[a] First (or early) or second (or late) clutches were reported, and the results portrayed for first (I) and second (II) clutches. [b] Range. [c] Mean. Hatch success = #nestlings hatched/#eggs. Fledgling survival = #fledged/#hatched (until day 15–20, depending on the study). Nest success = #fledged/#eggs, also referred to as egg: fledgling ratio.

Year, location and hatch date had no effect on clutch size. Egg masses were significantly lower in 2012, likely because eggs were weighed by an inexperienced operator using a different scale during that year; therefore all 2012 egg masses were removed, and the data re-analyzed. Egg mass was significantly different among locations, but not year or HDJ, with Inglewood (IN) and Montgomery (MG) having significantly lighter eggs (B=-1.224; 95%CI: -2.054, -0.393; p=0.004 and B=-1.768; 95%CI: -2.645, -0.891; p<0.0001, respectively, Figure C-8).

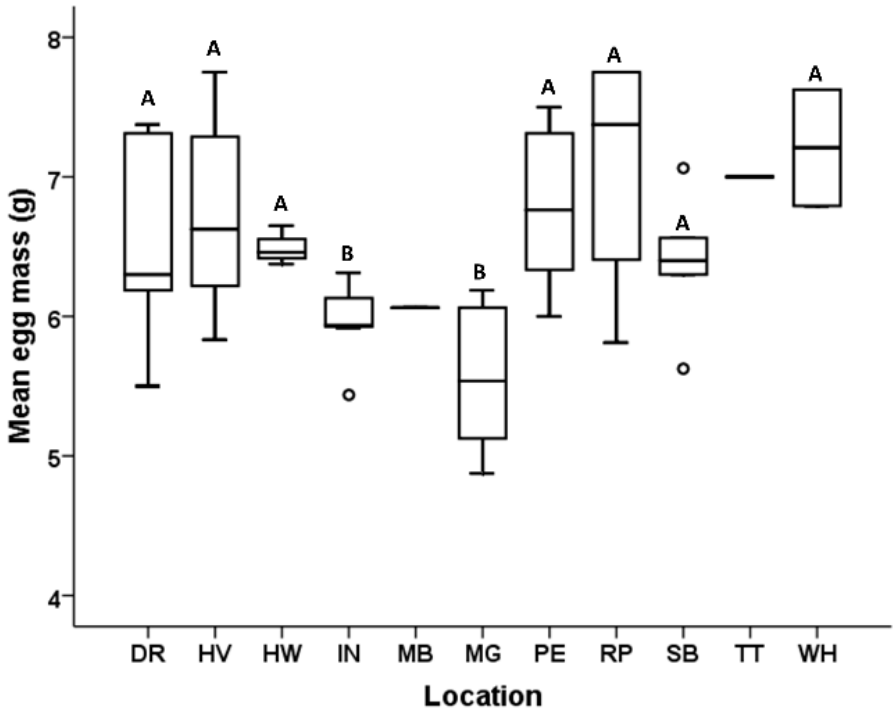

Figure C-16. Mean egg mass for European starlings by location in Calgary, AB. Different letters show statistically significant differences. MB and TT had only one sample each and could not be included in the statistical analyses. Location acronyms per Table 3-1.

Daily precipitation during the week before hatch was not correlated with precipitation during the week after hatch (p=0.094). Year accounted for 53.2% and 28.6% of the variation in precipitation during the week before and after hatch, respectively (p<0.0001). HDJ was only correlated with precipitation after hatch (r_S=0.391, p=0.002, n=61), accounting for 8% of the variation (p=0.031). In 2013, there was significantly more precipitation during the weeks before and after hatch (mean ± SEM, 9.82±1.01 mm and 3.55±0.71 mm, respectively), than 2012 (1.54±0.72 mm and 3.35±0.51 mm), 2014 (0.88±0.51 mm and 1.15±0.36 mm) and 2015 (2.69±0.57 mm and 1.02±0.40 mm, Figure C-9). The increased precipitation corresponded with the poorest year for data collection, with only 6 nests established, 5 producing offspring. Interestingly, Williams *et al.* (2015) also found 2013 to be an anomalous year in their starling population in Langley, British Columbia, 642 km southwest of Calgary, with the poorest nestling survival of their 13-year study (2002–2014).

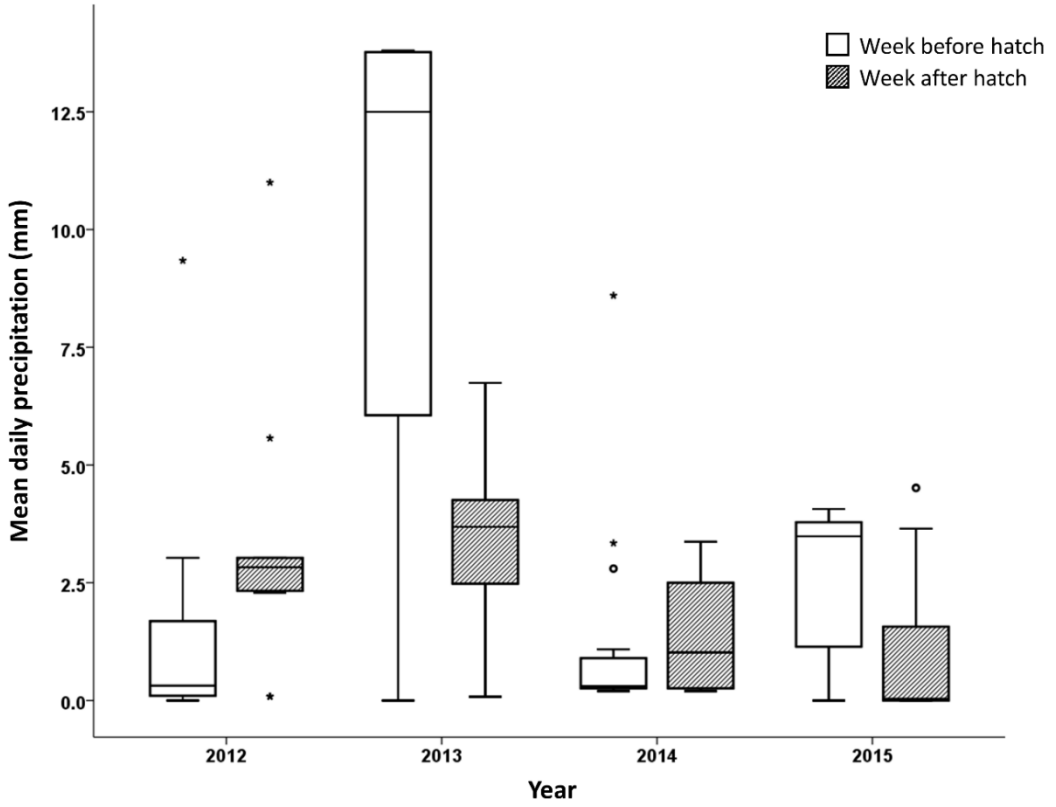

Figure C-17. The mean daily precipitation (rain + snow) in Calgary for the weeks before and after hatch of 2012, 2013, 2014 and 2015, demonstrating the difference among years.

Mean daily precipitation during the week before hatch is correlated with 9-day-old nestling SMI (r_S=0.208, p=0.009, n=155), with nestlings being in better condition approximately two weeks after there has been good rainfall. Rainfall may cause an increase in terrestrial invertebrates or fruit two weeks later, possibly causing this improvement in body condition.

Nestling mortality is significantly correlated with average temperature during the week after hatch (r_S= -0.367, p=0.004, n=61), with colder temperatures during nestlings' first week associated with higher mortality.

Tarsal length provides a measure of nestling skeletal growth, while wing chord is a measure of developmental maturity and readiness for flight. Table C-3 shows these characteristics of nestling starlings in Calgary, and their body mass and condition, at 9- and 15-days of age.

Table C-10. European starling nestling growth and development in Calgary (2014–2015, except where specified otherwise)

		n	Mean ± SEM	Range
Day 9	Body mass (g)	159	60.7 ± 0.6	33.5–75.5
	Wing chord (mm)	160	47.0 ± 0.5	23–61
	Tarsal length (mm)	156	28.9 ± 0.1	25–31
	Scaled mass index	155	63.7 ± 0.4	51.0–77.9
Day 15	Body mass (g)	133	69.7 ± 0.7	36.5–85.5
	Wing chord (mm) [a]	61	84.6 ± 0.6	72–95
	Tarsal length (mm)	128	30.1 ± 0.1	28.2–33.2
	Scaled mass index	129	66.0 ± 0.7	39.0–90.2
Change in SMI between day 9 and 15 [‡]		125	2.3 ± 0.9	-20.4–37.0

[a] 2015. [‡] Difference between SMI on day 15 and the SMI on day 9

Smith and Bruun (1998) reported 14-day nestling masses of 55.2–80.3 g, with a mean of 69.2 g (n=66), for a population in Sweden. Fifteen-day-old nestlings from a reference population of nestlings weighed 65.07 ± 0.83 g (mean ± SEM, n=98), in Illinois, USA (Arenal *et al.*, 2004). Therefore, it is evident that nestlings from this study in Calgary, Alberta, are comparable to those studied elsewhere in the world.

When nestling morphometric data from 2014 and 2015 were analyzed together, hatch date was significantly correlated with day 9 and 15 wing chords (r_S=0.216, p=0.006, n=160 and r_S=0.469, p<0.0001, n=61, respectively), and day 15 tarsal length (r_S= -0.240, p=0.006, n=128), but not with day 15 body mass or condition (p>0.060), with nestlings hatched later in the season tending to have longer wings (closer to fledging) at 15 days of age, and shorter tarsi than those that hatched earlier (Figure C-10, part A and B). This suggests that later broods prioritize development (flight) over size, favouring short term survival and readiness for migration before winter, and supports findings in other species (Birkhead and Nettleship, 1982, Cooch *et al.*, 1991, Sedinger and Flint, 1991, Ydenberg *et al.*, 1995, Lepage *et al.*, 1999). However, these findings contrast with those of

Grüebler and Naef-Daenzer (2010), who found that second-brood barn swallows' primary feather growth decreased with hatch date.

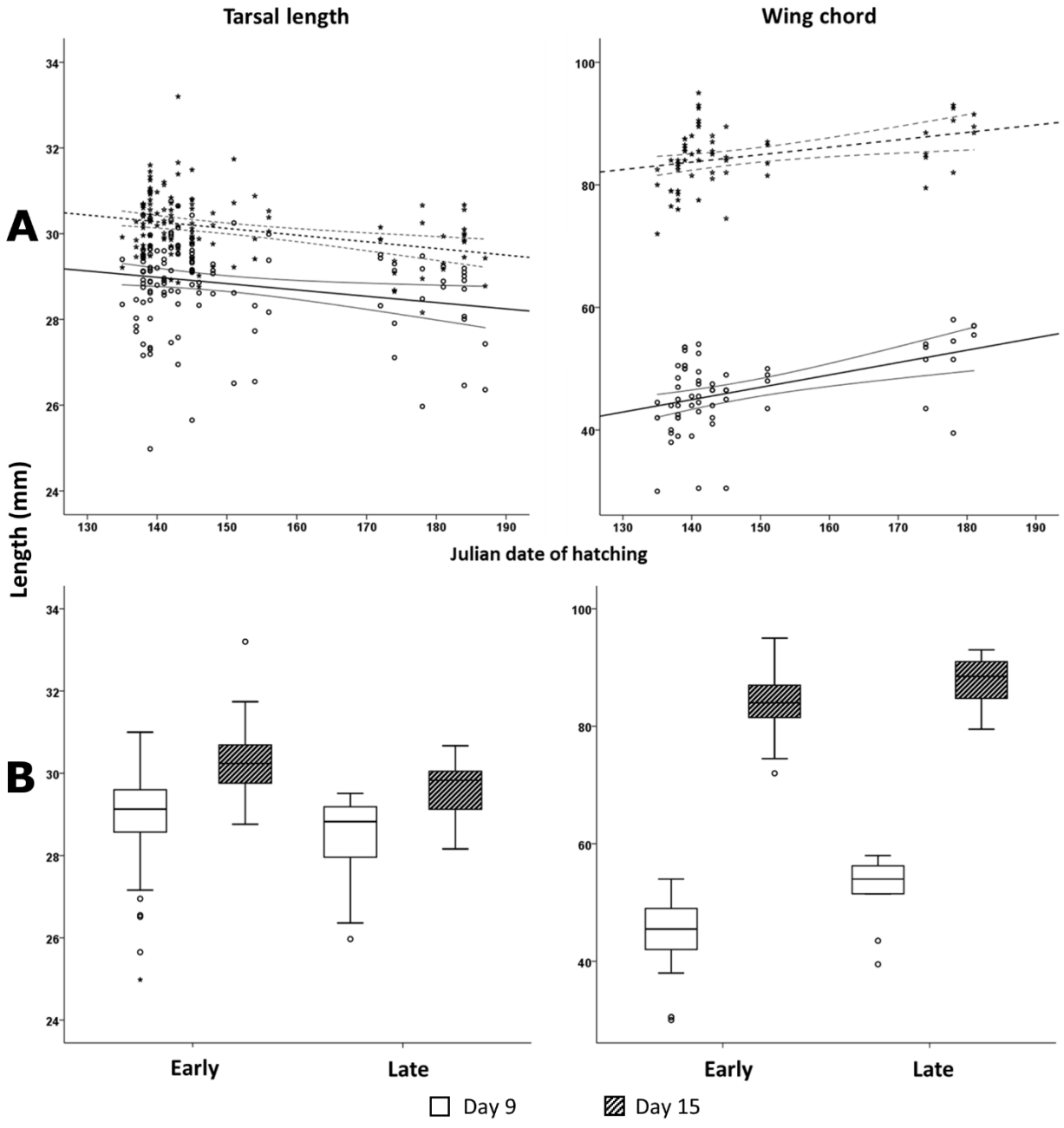

Figure C-18. Nestling growth and maturity by hatch date (A) and season (B). Day 9 and 15 nestlings are evaluated separately, with the solid and dashed lines indicating day 9 and 15, respectively, in graph A.

Nestling diet in 2015 varied over the summer: hatch date was significantly correlated with the percentage of stomach contents made up of vegetation (r_S= -0.511, p=0.004, n=30), and berries (r_S=0.746, p<0.0001, n=29), with worms and other invertebrates consumed at similar rates throughout the season. Late-brood nestlings tended to eat less vegetation (i.e., grass) and more berries than early nestlings (Figure C-11). Nestling scaled mass index (day 15) was not related to hatch date (p=0.569), but was positively correlated with the percentage of invertebrates in the nestlings' stomachs (r_S=0.421, p=0.023, n=29), suggesting that insects like grasshoppers play a key role in starling growth and development in Calgary, throughout the breeding season.

These results, while interesting, need to be interpreted with caution: for the small sample size, the preliminary sampling methods that could be refined with further use, and since they only represent nestlings from a single year. Given that starlings have an average retention time of approximately 18–20 minutes per gram of food (Levey and Karasov, 1994), the contents of the stomach represents only the most recent meal and may not be representative of the diet of the nestlings while in the nest. However, it would be interesting to include this data in any future studies.

Diet composition was not different between riverine and non-riverine areas (p>0.245), and groomed and natural areas (p>0.710).

At 15 days of age, nestlings from upland nests tended to be in better condition than nestlings from parks near rivers (SMI, mean ± SEM: upland, 71.07±1.76 and riverine, 66.08±1.74; B=4.988, 95%CI: 1.778, 8.198; p=0.002). Brood size was almost significant for 15-day-old nestling body condition for a brood size of 1, where the nestling was in better condition than all other brood sizes (B=15.451, 95%CI: -0.481, 31.383; p=0.057).

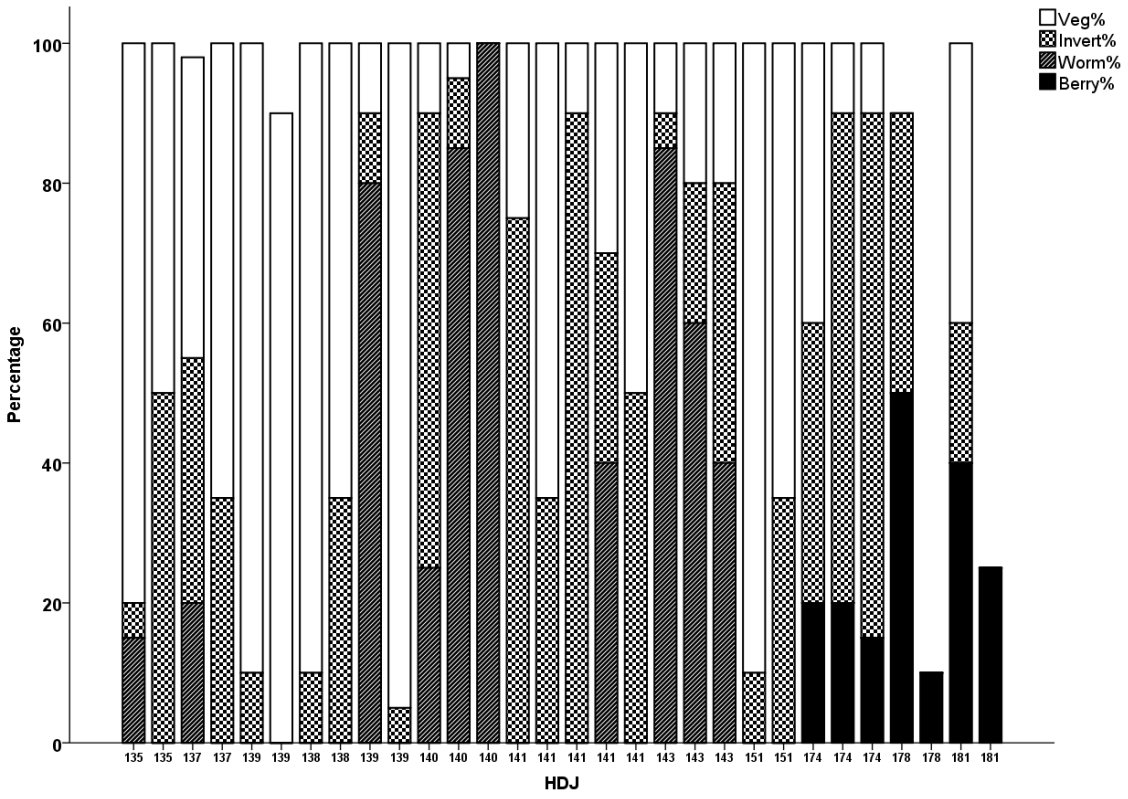

Figure C-19. Relative contribution of vegetation, invertebrates, worms and berries in stomach contents by hatch date in the summer 2015. The points that don't add up to 100% are a result of missing data.

C.4. Some Notes on Confounders

Adverse weather is known to affect nestling growth and survival (Gentes *et al.*, 2006), and may overwhelm any subtle effects due to air pollution. Where possible, these need to be accounted for in analyses to tease out the relative contribution of weather on biological responses. Often, weather may be linked to annual or seasonal variation—the relationship between these should be checked carefully to prevent autocorrelation confounding statistical analyses.

Differential parent quality and genetics could play a significant role in influencing nestling growth and survival (Martin, 1987); however, without collecting data on the parent birds (size, condition, genetics) the effect of parent quality should be accounted for indirectly. Statistical models should regard the clutch as being the individual subject, with sibling data included as within-subject or

repeated measures to account for the lack of independence between sibling data. Reproductive success could be regarded as both a proxy for parental quality and a biomarker of exposure. Depending on sample size and the predicted effect size, using reproductive success as a proxy for parent quality may overly-complicate statistical models, reducing their sensitivity for detecting subtle changes.

Differences in diet quality and quantity would be expected to affect nestling fitness (e.g., body condition, growth, and survival) (Martin, 1987, Eeva *et al.*, 2009), and could be detected as a difference in body condition between nests with more or less experienced parents, or locations with varying diversity in food choice. As opportunistic feeders, starlings preferentially eat terrestrial invertebrates, but will readily subsidize diet with earthworms, berries, fruit or food from human waste (Moeed, 1980, Tinbergen, 1980). The composition of the diet may vary based on seasonal availability of food types, such as insect larvae versus fruit or berries, and may affect nestling growth and survival (Martin, 1987).

C.5. Conclusions

This population of starlings has similar reproductive timing and success when compared to other relatively northern, North American populations. There is nothing to suggest that there is anything adversely affecting the reproduction or survival of starlings in Calgary from a population-level, and therefore this population should be suitable for use as sentinels for the monitoring of air pollution toxicology. However, since all birds monitored during this study were in a natural setting, the effects of background, unmeasured exposures endemic to urban environments may preclude the conclusion that this population is completely 'normal', or untouched by anthropogenic effects.

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APPENDIX D: ENCLOSURE DESIGN FOR FLOCK-LEVEL, CHRONIC EXPOSURE OF BIRDS TO AIR CONTAMINANT MIXTURES

D.1. Abstract

The objective of this study was to design an enclosure suitable for studying the ecotoxicological effects of vehicle emissions on groups of wild birds without compromising welfare. Two, adjacent enclosures sheltered from sunlight, wind and rain, were bird-proofed and wrapped with thick polyethylene sheeting. Emissions were directed into the exposed enclosure from the exhaust of a light-duty gasoline truck, using flexible, heat-proof pipe, with joins sealed to prevent leakage. During active exposure, the engine was idled for five hours per day, six days per week for four weeks. Fans maintained positive pressure (controls) and negative pressure (exposed), preventing cross-contamination of enclosures and protecting investigators. Four sets of passive, badge-type samplers were distributed across each enclosure, measuring nitrogen dioxide, sulfur dioxide and volatile organic compounds (NO_2, SO_2 and VOCs, respectively), and were complemented by active monitors measuring VOCs and particulate matter (2.5 µm diameter, $PM_{2.5}$). We found that the concentrations of NO_2, SO_2 and $PM_{2.5}$ were not different between exposed and control enclosures. Volatile organic compounds (e.g., benzene, toluene, ethylbenzene and xylenes) were approximately 6 times higher in the exposed chamber than control (13.23 $\mu g.m^{-3}$ and 2.13 $\mu g.m^{-3}$, respectively). In conclusion, this represents a successful, practical design for studying the effects of sub-chronic to chronic exposure to realistic mixtures of vehicle exhaust contaminants, in groups of birds. Recommended modifications for future research include a chassis dynamometer (vehicle treadmill), to better replicate driving conditions including acceleration and deceleration.

D.2. Introduction

Air pollution is a growing global concern; designated a major environmental health risk by the World Health Organization (WHO, 2016), in 2012, outdoor air pollution contributed to approximately 3.7 million premature deaths globally. The WHO Air Quality Guidelines recommend threshold concentrations for particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2) and sulfur dioxide (SO_2) to reduce air pollution-related mortalities.

Automobile (on-road) traffic is a major source of air pollution, particularly oxides of nitrogen (U.S. EPA, 2016a) and volatile organic compounds, notably benzene, toluene, ethylbenzene and xylenes (collectively referred to as BTEX). The concentrations of traffic-source pollutants tend to be highest alongside major roads, and decrease with distance from the road. In many urban areas, homes near roads may have higher pollutant concentrations than the city-average, exposing the residents to elevated health-risks (Hoek et al., 2002, Perez et al., 2013).

There are two principal methods for studying the effects of exposure to air contaminants in animals: laboratory-based inhalation toxicology and ecotoxicology field studies. Laboratory studies typically involve the high-concentration, short duration exposure of traditional research species (such as rats, mice and guinea pigs) to single compounds or limited mixtures (Hinners et al., 1966), and require significant experience in engineering and chemistry. Field studies investigate the effects of exposure to common environmental mixtures of contaminants using diverse species including small- (Borras et al., 1998) or large (Waldner et al., 2001) mammals, birds (Cruz-Martinez et al., 2015a), or multi-species comparative studies (Llacuna et al., 1993), and require extensive biological expertise, including knowledge of physiology and population-ecology. Epidemiological research into the effect of air pollution on human health are a third method of studying the toxicology of air pollutants and will not be discussed here. While experimental, lab-based studies and field studies each have distinct advantages, the disadvantages can complicate our understanding of the consequences of exposure to realistic pollutant mixtures in wildlife species. This study controls many of those disadvantages, while retaining the advantage of ensuring environmentally relevant results (Table D-1).

Table D-11. Characteristics of laboratory and field studies, in relation to this experimental enclosure design

	Laboratory exposures	Field-based exposures	This experiment
Advantages	Controlled exposures Genetically inbred animals Good for determining toxic mechanisms of action Good for answering a specific question	Environmentally relevant exposures Exposure to mixtures Outbred populations Wildlife or domestic species	Many the advantages of the previous 2 columns: Controlled conditions with fewer confounding factors Genetically diverse population of wild birds (or inbred animals, depending on the goal of the study) Real pollutant mixture profile
Disadvantages	Frequently high-concentration, short-duration experiments Single-contaminant exposures Limited to typical laboratory species	Diverse populations (age, sex, genetics) Many uncontrolled, confounding factors (e.g., weather, diet, exposure to unaccounted toxicants)	Avoids most of these disadvantages The necessity to meet Occupational Health & Safety requirements can restrict experimental conditions

The highly efficient avian respiratory system and sensitivity to inhaled toxicants makes birds ideal sentinels for the effects of air pollutants on ecosystem health (Brown et al., 1997). Field studies using birds have identified biomarkers of exposure to airborne contaminants in urban and rural settings (McArn et al., 1974, Eeva et al., 2000, Herrera-Dueñas et al., 2014); however, confounding factors can obscure subtle effects and complicate interpretation of results (North et al., 2017). Olsgard and Smits (2008) designed a specialized chamber for the controlled exposure of birds to pollutant mixtures, which has been used successfully in several studies using American kestrels and Japanese quail (Olsgard et al., 2008, Cruz-Martinez et al., 2015b). However, the birds can only

be maintained in the exposure chambers for brief periods (~ 2 $h.d^{-1}$), limiting the study of sub-chronic or chronic exposures.

European starlings (*Sturnus vulgaris*) were selected for this experiment since they are a well-studied, prolific species that frequently co-habits urban environments with human residents, and have been used as a sentinel species in several environmental toxicology studies (Trust et al., 1994, Wolfe and Kendall, 1998, Parker and Goldstein, 2000, Arenal et al., 2004, Zahara et al., 2015). To support the results of field studies (e.g., North et al. (2017), Chapter 3), an 'inhalation chamber' was required that permitted chronic exposure to airborne contaminants in a manner consistent with what is known of starling welfare and behavior. In a review of research using captive starlings, Asher and Bateson (2008) recommend they be group-housed to decrease stress, in enclosures providing for ample horizontal flight-space, natural substrate for gleaning, branches of varied width for perching, and water baths.

Since motor vehicles form the greatest mobile source of contaminants in urban areas, ideally, an experiment designed to study the toxicology of these contaminants should have a vehicle as the source of emissions. For the contaminant profile to be relevant to any specific city, the investigator should select a 'typical' vehicle, such as large or small vehicle, with a two- or four-stroke engine, fueled by gasoline, diesel, or compressed natural gas.

Active, real-time air samplers are the gold-standard for air quality monitoring. However, they are expensive and require a high level of expertise to ensure reliable data collection and interpretation. Additionally, active samplers need regular calibration and maintenance during use to ensure accuracy and thus are not generally practical for ecotoxicology research. In contrast, passive, badge-type samplers are practical and user-friendly, but still require validation using duplicate samplers and field blanks to confirm accuracy and correct handling. Because of the inherent complexity of studying effects from, or measuring levels of, air contaminants, simultaneous, complementary methods of measuring air contaminants is the preferred option.

The objectives of this study were to design an exposure chamber which enabled: i) sub-chronic to chronic exposure of ii) groups of birds, to iii) realistic mixtures of pollutants from combustion

engines (a vehicle, in this case), iv) without undue stress to the birds or risk to research personnel, and v) exposing the control birds to the same background noise and investigator presence, vi) while at the same time enabling measurement of the contaminants.

D.3. Methods

D.3.1. Structural components & design

Pollution source

The emission source was a well-maintained 2010-model Toyota Tundra with a catalytic converter, defined by the U.S. EPA as a light-duty gasoline truck (U.S. EPA, 2008). The truck exhaust was connected to the 'exposed' enclosure using a 2 m long, heat-proof, flexible pipe with a 10 cm internal diameter that fitted snugly over the vehicle exhaust. The vehicle was started approximately 30 minutes before being connected to the enclosure to warm up the engine. The fuel used was commercially available, regular (87 octane) Canadian gasoline.

Enclosure design & materials

Two enclosures (length x breadth x height, 6 m x 2 m x 2 m, volume = 24 m^3) were constructed 2 m apart under a solid canvas marquee, with an enclosed atrium serving as entrance for both (Figure D-1). The enclosures were sealed to prevent contaminant leakage using semi-clear, heavy-duty (150 µm) polyethylene sheeting with overlap at joins and floor. A negative pressure was maintained in the exposed enclosure using a 0.5 m, 3-speed box fan on its lowest setting (< 2,500 CFM, or < 1.2 $m^3 s^{-1}$). The air in the control enclosure was maintained at ambient conditions using the positive pressure provided by two 0.5 m high-velocity fans (3,900–6,100 CFM, 1.8–2.9 $m^3 s^{-1}$). The visual confirmation of positive and negative pressure was sufficient for this study, making more formal measures of static pressure unnecessary. The enclosures and central atrium were bird-proofed on the inside using soft, fine-mesh landscaping drape.

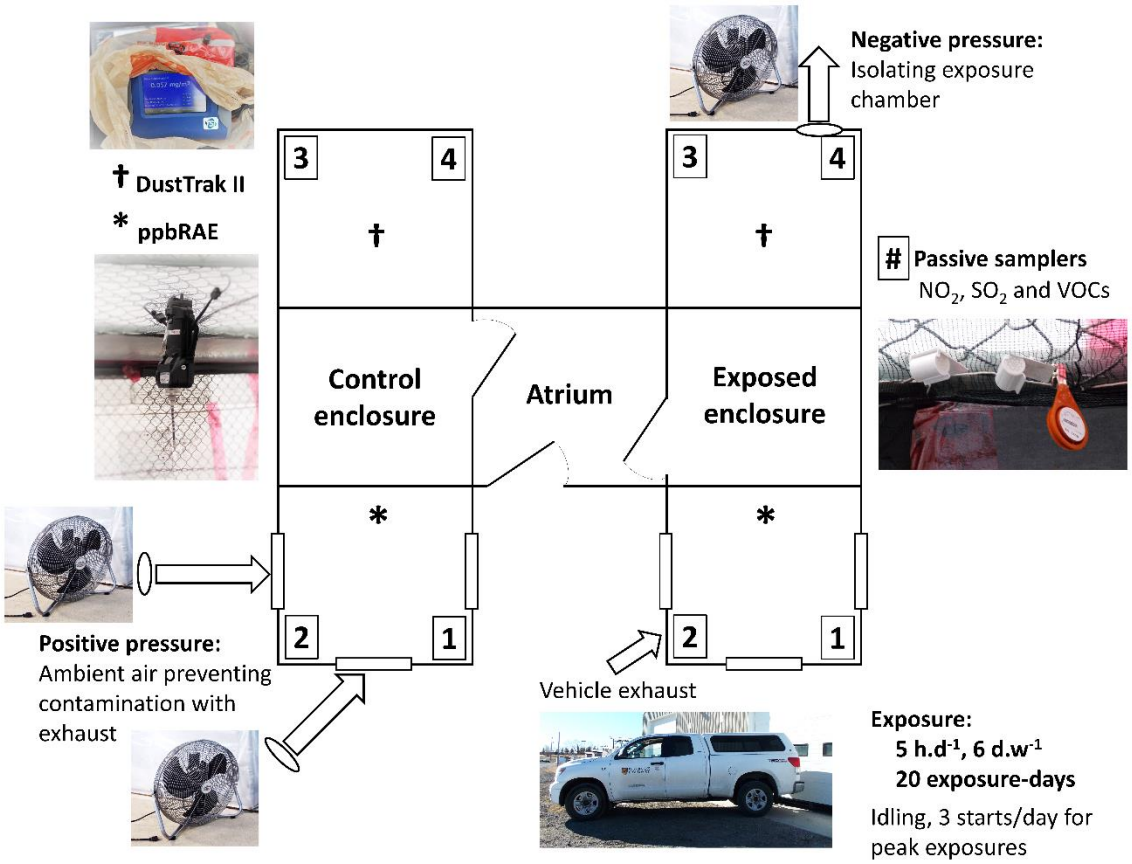

Figure D-20. Schematic design of the experimental enclosure described by this study, depicting the structural layout (open rectangles = windows, crescent lines = doors, thin lines = enclosure walls covered and sealed with polyethylene sheeting), positions of the monitoring equipment, emissions source and airflow.

D.3.2. Operation

Animals

Thirty adult, wild-caught European starlings were housed in each enclosure (control and exposed), providing 0.8 m^3 per bird (European guidelines for group-housed birds: 0.33 m^3 per bird; Bateson and Feenders (2010)). Environmental enrichment included ropes, chains and branches of varying thickness, water baths, natural mulch substrate and spaces to hide (refugia). Horizontal flight-space and floor area for gleaning behaviour exceeded recommended requirements.

Air quality monitoring

Passive, badge-type samplers were used to measure the concentrations of NO_2 and SO_2 (Ogawa USA, Pompano Beach, Florida) and VOCs (3M^{TM} Organic Vapor Monitor 3500, USA). Samplers were deployed in the morning of the first day of exposure and taken down 21 (21.56 ± 0.42) days later. Two additional VOC monitors were deployed in the exposed enclosure only for the last ten days to validate the consistency of exposure and as backup in case the first batch of samplers were saturated. The samplers were numbered and arranged as shown in Figure D-1. Passive samplers were supplied and analyzed by the Alberta Centre for Toxicology according to manufacturers' protocols (Appendix A).

Passive samplers provide an integrated, averaged measure of pollutant exposure for the duration of deployment. To approximate the BTEX concentrations during active exposure, the total burden of BTEX was calculated using the equation below:

$$[BTEX_{ACTIVE}] = \frac{\{([\mathrm{BTEX}_{\mathrm{exposed}}] \times TIME_{DEPLOYED}) - ([BTEX_{CONTROL}] \times TIME_{DEPLOYED})\}}{TIME_{ACTIVE}}$$

where $\mathrm{TIME}_{\mathrm{ACTIVE}}$ = minutes of active exposure and $\mathrm{TIME}_{\mathrm{DEPLOYED}}$ = minutes of full deployment. To account for the time required to clear the air after active exposure, an extra hour was added for this calculation ($\mathrm{TIME}_{\mathrm{ACTIVE}}$ = 6 hours: i.e., 5 hours' exposure + 1-hour clearing time).

Particulate matter smaller than 2.5 µm diameter ($PM_{2.5}$) was measured continuously in both enclosures using DustTrak monitors (DustTrak II Aerosol Monitor 8530, TSI®, Minnesota, USA). The data log was downloaded daily from the instruments after the exposure period, using TrakPro software, and transferred to Microsoft Excel for interpretation.

A real-time, home-use carbon monoxide (CO) monitor (Kidde Carbon Monoxide detector C3010-D-CA, Kidde Canada) was installed to ensure that neither the birds nor the investigators are exposed to dangerous CO levels.

Exposure, air flow & equilibration

Concentrations of VOCs measured during a pilot study were used to determine how to achieve the desired concentrations (i.e., ideally 50-100x ambient levels found in Calgary during May and June, see North et al. (2017)). The extraction fan speed controlled the dilution of exhaust and was regulated by stringent University of Calgary Occupational Health and Safety guidelines, which required extensive extraction to maintain visible negative pressure.

The truck was run for 5 hours per day, 6 days per week for a total of 20 exposure-days (100 hours). To provide peak exposures and promote the accumulation of contaminants in the enclosure, the truck was stopped and restarted immediately, three times during each 5 hour period.

D.3.3. Validation

Air flow

Air mixing within the enclosures, enclosure seal, and clearing time was visualized using theatrical smoke generators. Fans blowing ambient air (control) and the inlet of the exhaust pipe (exposed) were aligned perpendicular to the longitudinal axis of the enclosures to promote mixing (Figure D-1), which would also be facilitated by the birds' movement within the enclosures.

Exposure concentrations

Additional experiments were used to validate VOC and NO_2 exposures (without birds). VOC samplers were exposed for 6, 12 and 18 hours (all exposures exceeded the recommended minimum deployment period) to obtain time-concentration curves. To validate NO_2 concentrations, 18 Ogawa samplers and two samplers from an independent manufacturer (Maxxam Analytics Inc., Edmonton, AB) were suspended in an enclosure (Figure D-2), for one week (six days with vehicle running and one day without), following the experimental exposure protocol. Control samplers (one Maxxam and two Ogawa) were suspended in a ~ 50 m distant, upwind, sheltered location to measure ambient NO_2.

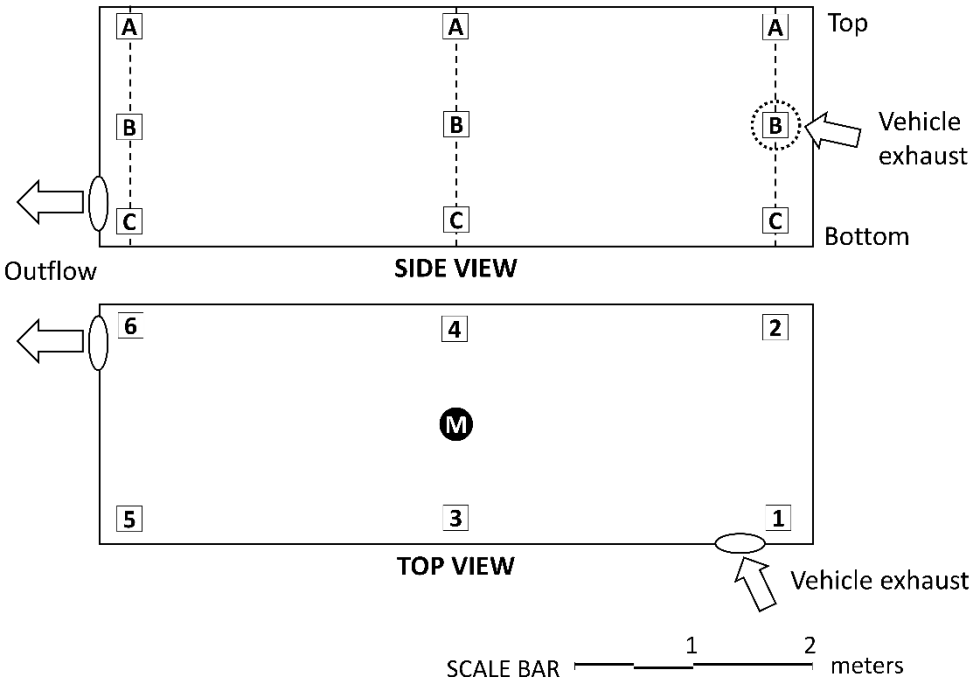

Figure D-21. Passive sampler set-up for NO_2 validation. Boxes (A, B & C, and 1–6) represent passive samplers, the black circle with a white 'M' inside it represents the position of the duplicate Maxxam samplers.

Field blanks (duplicate) were included; all reported results are blank-corrected except Maxxam samplers, which were only included for comparison with Ogawa samplers. The investigators did not include a blank for Maxxam samplers for financial reasons.

Alternative methods

Real-time VOC monitors (ppbRAE 3000, RAE Systems, by Honeywell International Inc., NJ) were run in duplicate to support the differences in concentration between control and exposed enclosures, and changes in concentration over time. These monitors measure VOCs as 'isobutylene units' which are not comparable to the passive samplers, but were used to verify the enclosure seal (ensuring investigators' safety) and accumulation of VOCs during exposure.

D.3.4. Statistical analyses

To compare the air pollution concentrations between the control and exposed enclosures, the mean, standard deviation and range of the passive sampler measurements were calculated and compared to each other, to the ambient concentrations in the city of Calgary (measured during the summers of 2013–2015), and to regulatory guidelines for the compounds (e.g., those of the U.S. EPA). Statistical significance (p-values) were not calculated due to the small sample sizes and repeated measures.

D.3.5. Safety precautions and Animal Care

In accordance with the University of Calgary's Department of Occupational Health and Safety, investigator and animal care personnel health were protected by implementing the following:

- A household carbon monoxide (CO) monitor was installed outside the enclosures where the investigators were, to ensure that CO concentrations did not exceed safety guidelines. No CO was detected for the duration of the experiment.
- VOC sniffers (handheld ppbRAE) were used during the initial period of exposure to check for leaks in the polyethylene-wrapped enclosures.
- The effective seal provided by the polyethylene sheeting was also checked using a theatrical smoke generator to visualize the gas dispersal within and escaping the enclosures, and finally, positive (control enclosure) and negative (exposed enclosure) pressures generated by the fans were evident through the outwards or inwards billowing of the polyethylene wrap.
- Personnel were not permitted to enter the enclosures during active exposure, and signs describing the nature of the study were placed at the entrance.

All housing, handling and procedures for the birds used during this experiment complied with University of Calgary Animal Use Protocol AC15-0176.

D.4. Results

D.4.1. Particulate matter (2.5 µm), nitrogen dioxide & sulfur dioxide

We found no difference in $PM_{2.5}$ concentrations between enclosures (control: 9 ± 1.82 µg.m^{-3}, exposed: 11 ± 2.34 µg.m^{-3} (mean ± SD)), with values well below EPA guidelines (35 µg.m^{-3} for 24-hour exposure, U.S. EPA (2016b)). The $PM_{2.5}$ concentrations measured in this experiment are likely caused by the birds, investigators and animal care personnel disturbing bedding material, thus suspending dust particles in the air, rather than the vehicle exhaust. Concentrations of NO_2 and SO_2 did not vary between control and exposed enclosures and were comparable to those measured in Calgary (Figure D-3), although for NO_2, both groups were significantly higher than blank samplers.

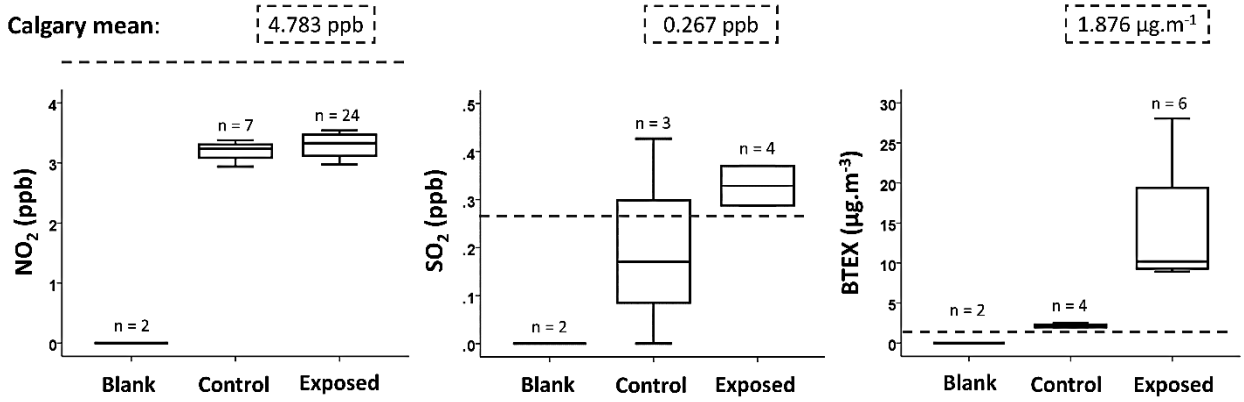

Figure D-22. Concentrations of NO_2, SO_2 and BTEX measured during the experiment, for blank samplers, control and exposed enclosures. Results for individual compounds (benzene, toluene, ethylbenzene and xylenes) follow the same pattern as BTEX. Control concentrations represent average ambient conditions in the city of Calgary, as shown by the horizonal, dashed lines (mean concentrations measured during the summers of 2013–2015).

D.4.2. Volatile organic compounds

BTEX

Benzene, toluene, ethylbenzene and xylenes (integrated as BTEX) concentrations in the control enclosure (mean: 2.13 µg.m^{-3}, range: 1.94–2.52) were comparable to those measured in Calgary during 2013–2015 (mean: 1.84 µg.m^{-3}, range: 0.81–3.03, Figure D-3 (North et al., 2017)), while concentrations in the exposed enclosure were on average, 6.2 times higher (mean: 13.23 µg.m^{-3}, range: 8.93–28.03). During active exposure, BTEX was approximately 62 µg.m^{-3} (Figure D-4).

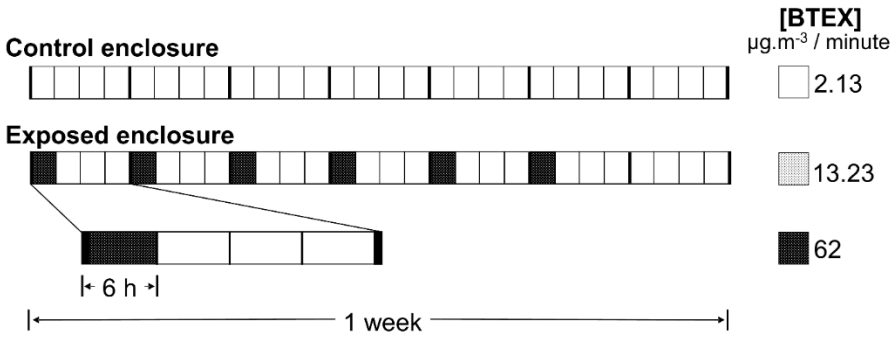

Figure D-23. A schematic of a typical week during exposure, with BTEX concentrations in control (above) and exposed (below) enclosures. Dark grey blocks represent the period of exposure (5 hours active exposure + 1 hour for complete air change).

BTEX concentration in the exposed enclosure had a gradient from inlet (10.75 and 28.03 µg.m^{-3}) to outflow (8.93 and 9.68 µg.m^{-3}), with the highest concentration being in the corner nearest the truck exhaust and the other three corners having similar concentrations. The concentrations of benzene, toluene, ethylbenzene and xylenes measured with each sampler in the control and exposed enclosures can be found in Table D-2.

Table D-12. Variation in BTEX concentrations within the enclosures

Contaminants (µg.m^{-3})		Benzene	Toluene	Ethylbenzene	*m,p*-Xylenes	*o*-Xylene	BTEX	BTEX/group
Exposed	E1	1.94	4.12	0.61	3.06	1.03	10.75	14.35
	E2	5.47	9.30	1.65	8.55	3.06	28.03	
	E3	1.56	3.23	0.54	2.69	0.90	8.93	
	E4	1.67	3.46	0.61	2.96	0.99	9.68	
Exposed (second batch)		3.08	4.63	0.50	3.73	1.04	12.98	12.11
		2.78	3.93	0.42	3.19	0.92	11.25	
Control	C1	0.22	1.02	0.09	0.54	0.11	1.98	2.13
	C2	0.33	1.34	0.11	0.61	0.13	2.52	
	C3	0.22	0.91	0.09	0.61	0.11	1.94	
	C4	0.25	0.98	0.11	0.63	0.13	2.10	

The codes (E or C, number) correspond with Figure D-2, where E(#) refers to the samplers deployed in the exposed enclosure for the first 19 days, 'second batch' to the pair of samplers deployed in the exposed enclosure for the last 10 days, and C(#) to the samplers deployed in the control enclosure.

Time-concentration curve

Accumulation and fluctuation of VOC concentrations within the exposed enclosure, and the difference in concentrations between exposed and control enclosures was evident from analysis of ppbRAE output (Figure D-5). The units are not comparable to those from the passive samplers, however, it is evident how the contamination changed over time, with peak exposures occurring as the vehicle was turned off and back on. Because of limitations of these data, they were not considered in the analysis of toxicologic responses. Their primary value was to check for accumulation or leaks of VOCs in the exposed chamber, for investigator safety according to Occupational Health and Safety requirements.

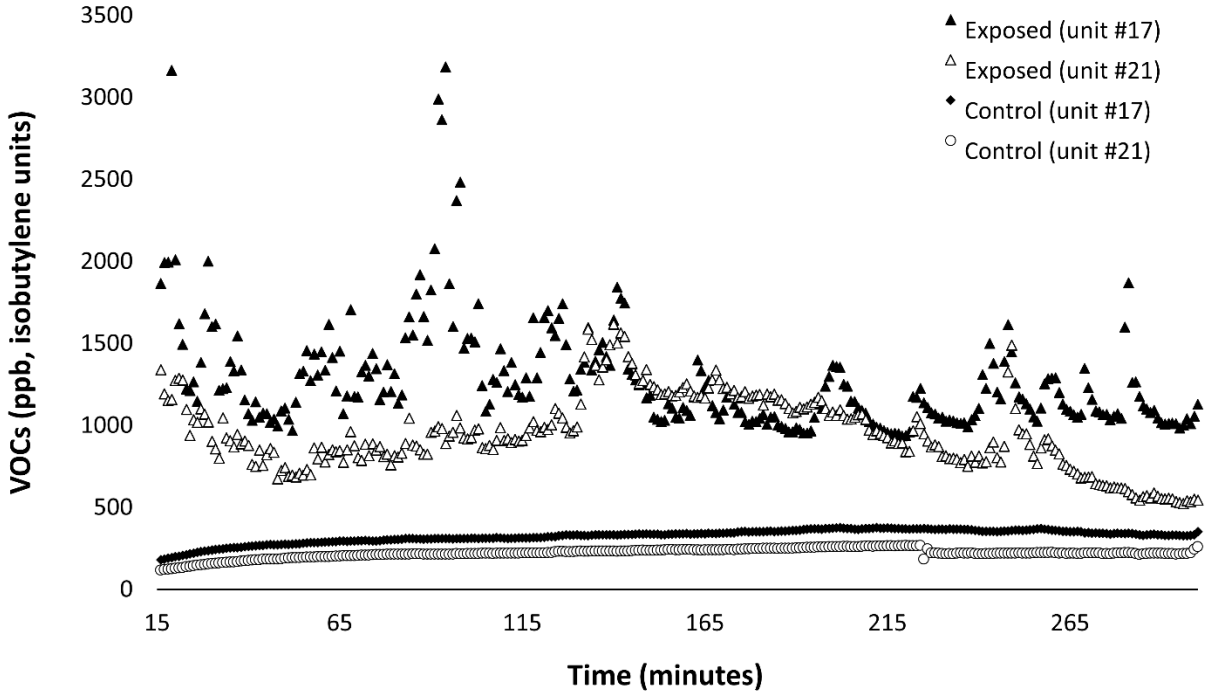

Figure D-24. VOC concentrations in exposed and control enclosures during active exposure, as measured using two handheld ppbRAE units (control tracings are the lower two lines). The data used to create this chart were trimmed (unreliable beginning and end exposures removed), and averaged for all days measured.

D.5. Discussion and conclusions

Birds are more sensitive to some inhaled toxicants than many mammals (Burrell et al., 1914, Brown et al., 1997, Pickrell, 2007), and yet regulatory air quality thresholds are largely based on data obtained from studies using laboratory rodents. Avian inhalation toxicology studies tend to focus on relatively higher-concentration, shorter-duration exposures (Olsgard et al., 2008, Cruz-Martinez et al., 2015b), partly for animal welfare reasons because exposure chambers are not suitable for chronic, group exposures. To understand and predict the potential effects of sub-chronic or chronic exposure to common airborne contaminants in free-living wild birds, a chamber designed specifically for the exposure of groups of birds to air contaminants for prolonged periods, is needed.

The enclosure design described here successfully meets these requirements, with only the emissions-source requiring some modification to optimize the protocol to achieve maximal environmental relevance.

While these BTEX results are low compared to other toxicology studies (Inoue et al., 1988, Olsgard et al., 2009) and environmental regulations (Table B-4), they represent plausible high-ambient exposure for urban environments (Fracasso et al., 2010, Miller et al., 2012). Additionally, the values represent an integrated exposure over the entire period, and do not indicate the peak levels during each 5-hour exposure. This is realistic of urban areas where peak traffic times lead to elevated concentrations of pollutants in the morning and evening (Calgary Region Airshed Zone (CRAZ), 2017).

While particulates emitted from gasoline engines are in the ultrafine range ($PM_{0.1}$, aerodynamic diameter of <100 nm, Ristovski et al. (1998)), and have been implicated in adverse health outcomes (Ibald-Mulli et al., 2002, Klot et al., 2002), most epidemiological research indicates $PM_{2.5}$ as being of greater concern (Schwarze et al., 2006), such that $PM_{2.5}$ is one of the 'criteria' air pollutants regulated by the U.S. EPA (2009). Therefore, despite the DustTrak missing the smallest particles, it met the needs of this study, where health outcomes were the focus, rather than the chemistry of vehicle emissions. Real-time monitors for VOCs and particulates were selected

based on availability, affordability and recommendations from colleagues in Health Canada. Other more sophisticated and sensitive sampling (such as grab samples for speciation of VOCs) were not feasible.

Since gasoline engines generally have lower SO_2 and $PM_{2.5}$ emissions than diesel engines, our use of a gasoline vehicle may explain the low concentrations of these compounds. Additionally, an idling engine may be operating at optimal oxygen-to-fuel ratios, with the catalytic converter removing most of the NO_2 from the exhaust and thus generating less emissions than if the vehicle were driving on-road (Tong et al., 2000, Frey et al., 2003). Interestingly, an EPA document that was used in the planning of this experiment, which described the emissions from an idling, light-duty gasoline truck, indicated that NO_x and VOCs should be emitted at similar rates (i.e., 4.043 and 4.065 $g.h^{-1}$, for VOCs and NOx, respectively, U.S. EPA (2008)), which is contrary to the findings of this study.

This study was conducted in a country with strict control of vehicle emissions, close regulation and stringent testing of vehicles before they are permitted on the roads, as well as a wealthier populace who can afford and maintain newer vehicles. These conditions are rarely met in most of the developing world, so such an experimental setup would be even more revealing using a 'typical' vehicle (such as an older, poorly maintained diesel vehicle or two-stroke motorcycle) found in many huge cities around the world. A second noteworthy point to consider before extrapolating results to developing regions, is the quality of fuel available in different countries, since fuel-quality will directly influence the quantity and profile of vehicle emissions. The strengths of this experimental design are its easy adaptation for relevance under different scenarios, tailored to the region of interest.

Smoke machines provide a valuable method of visualizing possible sites of leakage, but may not be entirely adequate to test for the homogenous dispersion of gas within enclosures, since the flow rate from the smoke machine may be higher than that of vehicle exhaust, and smoke may behave differently under experimental conditions than other chemical compounds. The uneven BTEX concentrations in the exposed enclosure (Table D-2) showed imperfect mixing of the air inside the

enclosures despite the fans, and the apparently adequate circulation visualized using theatrical smoke machines during the pilot study.

Laboratory and field experiments each have their own strengths and are valuable for answering different toxicological- or ecotoxicological questions. In this case we try to combine elements of the two methods (such as the natural mixture of gases, controlled environments etc.), however, for many larger environmental toxicology questions, the use of a combination of both methods together may be ideal to understand the effects of natural exposures. It is important for investigators to recognize and discuss the weaknesses of the approach chosen, honestly basing the results of the study in the context within which it was conducted.

Recommendations / future directions:

Using the same basic set-up and exposure protocol, the authors recommend the following modifications to improve the experimental process:

- The contaminant challenge would be more realistic if the emissions source was a vehicle under load, for example on an emissions-test treadmill or chassis dynamometer. This would allow investigation of different contaminant concentrations depending on the oxygen-fuel ratios.
- Lower exhaust dilution rate inside enclosures by reducing the extraction fan speed
- Include additional small fans inside the enclosures to promote complete mixing of the vehicle exhaust with the air.
- Combine a humidifier (control) or dehumidifier (exposed) to keep humidity more consistent.
- Use fine-gauge wire mesh (e.g., square weave, non-toxic metal coating, of gauge appropriate for species being studied) rather than soft material, despite the latter being easier to handle and more affordable. The birds could force the weave open, with several escaping in this manner over the exposure period.
- Video documentation would allow behavioural assessment (e.g., singing, feeding, grooming), as well as physiological responses to contaminant exposure.

The authors recognize the limitations of this study, yet the aim is to provide a basis for future ecotoxicological research, rather than regulatory toxicology. This is a practical experimental set-up, suitable for research into exposure of wild passerines to realistic mixtures of air pollutants. It can readily be adapted for chronic studies involving medium and longer-term exposures. It would also be possible to investigate the relative sensitivity of different avian species taking advantage of common song birds in various regions of the world, and by housing compatible species together in the exposure chamber (e.g., ground-dwelling species like quail or chickens) and passerines (house sparrows, finches, starlings, etc.).

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APPENDIX E: COPYRIGHT LETTERS

20 September 2017

To Faculty of Graduate Studies, University of Calgary:

We hereby give permission for Michelle A. North to include the manuscript: "**European starlings (*Sturnus vulgaris*) as sentinels of urban air pollution: a comprehensive approach from non-invasive to post mortem investigation**", published in the journal Environmental Science & Technology, as a chapter in her doctoral thesis, submitted to the Faculty of Graduate Studies, University of Calgary. We agree to the terms outlined in the University of Calgary Non-Exclusive Distribution License (http://grad.ucalgary.ca/files/grad/university-of-calgary-non-exclusive-distribution-licence.pdf), and are aware that University of Calgary theses are harvested by Library and Archives Canada, and may be submitted to ProQuest.

Sincerely,

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Title: European starlings (Sturnus vulgaris) as sentinels of urban air pollution: a comprehensive approach from non-invasive to post mortem investigation

Author: Michelle A. North, David Kinniburgh, Judit E. G. Smits

Publication: Environmental Science & Technology

Publisher: American Chemical Society

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To Faculty of Graduate Studies, University of Calgary:

I hereby give permission for Michelle A. North to include the manuscript: "**Biomarker sensitivity to vehicle exhaust in experimentally exposed European starlings**", published in the journal Environmental Science & Technology, as a chapter in her doctoral thesis, submitted to the Faculty of Graduate Studies, University of Calgary. I agree with the terms outlined in the University of Calgary Non-Exclusive Distribution License (http://grad.ucalgary.ca/files/grad/university-of-calgary-non-exclusive-distribution-licence.pdf), and are aware that University of Calgary theses are harvested by Library and Archives Canada, and may be submitted to ProQuest.

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Environmental Scientist | Ecotoxicologist
Azeral Environmental Sciences

13 October 2017

To Faculty of Graduate Studies, University of Calgary:

We hereby give permission for Michelle A. North to include the manuscript: "**Enclosure design for flock-level, chronic exposure of birds to air contaminant mixtures**", under review by the journal Toxicology Mechanisms and Methods, as a chapter in her doctoral thesis to be submitted to the Faculty of Graduate Studies, University of Calgary. We agree to the terms outlined in the University of Calgary Non-Exclusive Distribution License (http://grad.ucalgary.ca/files/grad/university-of-calgary-non-exclusive-distribution-licence.pdf), and are aware that University of Calgary theses are harvested by Library and Archives Canada, and may be submitted to ProQuest.

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