Impacts of urbanisation on birds

Disentangling the effects of multiple pollutants on avian behaviour and physiology

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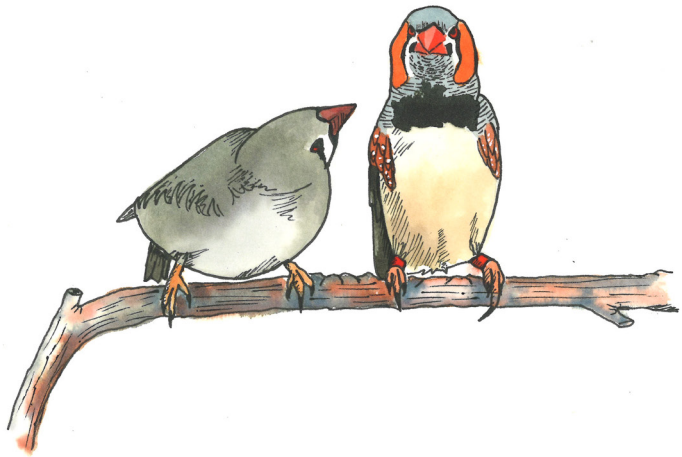

List of papers

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Abstract

Anthropogenic pollution is a pervasive feature of urbanisation, reaching into ecosystems worldwide and posing novel challenges to wildlife. Not surprisingly, differences in behaviour, and physiology, have been found between urban and rural populations. Most studies on anthropogenic impacts have so far either used a dichotomous approach, comparing urban-rural sites, or investigated impacts of just one stressor. However, urban environments create a complex matrix of co-occuring pollutants, leading to potentially complex interactive effects between stressors. We currently lack a deeper knowledge of the combined and single effects and the underlying mechanisms creating urban-rural phenotypic variation.

In this thesis, I investigated the single and combined effects of urban pollutants of avian behaviour and physiology. Specifically, I used the oxidative stress system, immune system, and plasma fatty acid composition, as the key physiological traits responding to human-induced environmental change. Urban pollutants of interest were artificial light at night (ALAN), anthropogenic noise, ozone and soot, and as human-influenced additional factors I looked at impacts of differential diets, and vegetation structure. I utilised full-factorial experimental exposure experiments in the wild and in the laboratory, and a correlative study in the wild, using wild and captive birds.

I found that ALAN exposure alone decreases activity and noise exposure alone decreases the proportion of birds found feeding. The combined exposure to these two pollutants led to a non-additive effect on the proportion of birds resting, with ALAN as the driving stressor. ALAN-exposed nestlings mounted a less strong immune response, with a reduction of melatonin levels being the likely mechanistic link to an impaired immune functioning. Simultaneous exposure to ALAN and noise increased levels of an important antioxidant, total glutathione, more than the additive effect from single pollutant effects would have estimated (positive synergistic effect).

Furthermore, I found that ozone is a potent pro-oxidant, negatively affecting antioxidant capacity, but we found no increased levels of oxidative damage due to ozone exposure. Soot exposure, on the other hand, did not affect avian oxidative stress status. Dietary ω6- and ω3-polyunsaturated fatty acids (PUFAs) modulated oxidative stress response to ozone exposure, but also act alone, with ω3-PUFAs decreasing non-enzymatic antioxidant capacity. Likewise, ω6:ω3 ratios of circulating PUFAs of wild nestlings are changed by human-influenced environmental factors, as well as their antioxidant capacity is negatively affected by air pollution and number of oak trees around their nest box. We also showed in this latter study, that using multi-stressor approach gives a more profound mechanistic understanding of phenotypic effects, then using a dichotomous comparison, which might obscure certain effects.

Overall, I show that pollutants affect behaviour and key fitness related physiological traits and that the combined exposure to multiple stressors can lead to unexpected non-additive effects. This highlights the need of a more thorough mechanistic understanding of multi-stressor effects. A deeper understanding of single and combinatory effects of anthropogenic stressors will help gaining crucial insight into populations and species resilience to environmental change, thereby targeted actions can be proposed to maintain biodiversity in cities and have a future development of sustainable cities.

Key words: Artificial light at night (ALAN), antioxidants, great tit, light pollution, LPS, multi-stressor, noise pollution, sensory pollution, troposheric ozone, urbanisation, zebra finch

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[*]Shared first authorship

Author contributions

I. AKZ, JR, AG and CI designed the experiment; AKZ and CI conducted the experiment; ES and AA analysed the videos; AKZ performed the statistical analysis. AKZ wrote the first draft; all authors edited and revised the manuscript; CI received funding.

II. AKZ, HW, JÅN and CI conceptualised and designed the study; AKZ, HW and AH carried out the field work; AKZ, AH, RM and VC performed the laboratory work; AKZ performed the statistical analysis; AKZ wrote the first draft; all authors edited and revised the manuscript; CI received funding.

III. AKZ, JR, AG and CI designed the experiment; AKZ and CI conducted the experiment; AKZ performed the laboratory work; AKZ performed the statistical analysis; AKZ wrote the first draft; all authors edited and revised the manuscript. AKZ and CI received funding.

IV. AKZ, JKJ, JR, AG, JÅN, CI designed the experiment; AKZ and JKJ conducted the experiment; AKZ, JKJ and LJG did the laboratory work; AKZ performed the statistical analysis. AKZ wrote the first draft of the manuscript; AKZ, JKJ, JÅN and CI edited and revised the manuscript; CI received funding.

V. JKJ, AKZ, JÅN and CI designed the study; JKJ and AKZ carried out the field work; JKJ mapped the territories; AKZ, CIX and JR performed the initial data management of the $PM_{2.5}$ data; JKJ carried out the laboratory analysis with help from AKZ, LGJ and SGD; JKJ analysed the data and drafted the manuscript with input from AKZ and CI; JKJ, AKZ and CI edited and revised the manuscript. CI received funding.

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Abstract

Anthropogenic pollution is a pervasive feature of urbanisation, reaching into ecosystems worldwide and posing novel challenges to wildlife. Not surprisingly, differences in behaviour, and physiology, have been found between urban and rural populations. Most studies on anthropogenic impacts have so far either used a dichotomous approach, comparing urban-rural sites, or investigated impacts of just one stressor. However, urban environments create a complex matrix of co-occuring pollutants, leading to potentially complex interactive effects between stressors. We currently lack a deeper knowledge of the combined and single effects and the underlying mechanisms creating urban-rural phenotypic variation.

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air pollution and number of oak trees around their nest box. We also showed in this latter study, that using multi-stressor approach gives a more profound mechanistic understanding of phenotypic effects, then using a dichotomous comparison, which might obscure certain effects.

Overall, I show that pollutants affect behaviour and key fitness related physiological traits and that the combined exposure to multiple stressors can lead to unexpected non-additive effects. This highlights the need of a more thorough mechanistic understanding of multi-stressor effects. A deeper understanding of single and combinatory effects of anthropogenic stressors will help gaining crucial insight into populations and species resilience to environmental change, thereby targeted actions can be proposed to maintain biodiversity in cities and have a future development of sustainable cities.

General introduction

The current time we are living in is marked by unprecedented anthropogenic impacts on the Earth's ecosystem, also known as the "Anthropocene" (Crutzen, 2006). Historically, the majority of the human population lived in rural areas, with relatively little impact on nature on a global scale, but especially over the last century, the pace of urbanisation and population growth has steadily increased (United Nations, 2019). In 1950, about 30% of the world's population lived in cities, today it is about 55%, and by 2050 trends predict that about 68% of the population will reside in urban areas (United Nations, 2019). The rapid expansion of urbanised areas across the globe (Seto et al., 2011) will drastically increase human impacts on ecosystems (Grimm et al., 2008; Vitousek et al., 1997).

The urbanised environment is accompanied by a potpourri of different stressors, posing novel challenges to urban-dwelling animals. We define "challenges" or "stressors" as stimuli that cause a quantifiable change from the "normal" state, whether it be physiological or behavioural, with negative or positive deviations (Coté et al., 2016). In the context of urban ecology, the normal state often refers to individuals living in natural or non-urbanised habitats, that is the habitats in which they have originally evolved. Thus, the urban environments exert powerful selective pressures (Hendry et al., 2016; Palkovacs et al., 2012; Salmon et al., 2021), which have to be met with an adequate response to avoid negative consequences. The nature of these responses can vary, ranging from phenotypic plasticity to genetic adaptations (Alberti et al., 2017a, b; Hendry et al., 2008; Partecke, 2014). Indeed, differences in morphology, physiology, behaviour and gene frequency and expression have been found between animals inhabiting urban and rural environments (e.g., Bonier, 2012; Hutton and McGraw, 2016; Isaksson, 2015; Munshi-South et al., 2016; Partecke et al., 2006; Shochat et al., 2006; Watson et al., 2017). While some species and/or populations seem to be able to utilise novel anthropogenic resources to their benefit, adapt to anthropogenic changes and thrive in urban environments (Callaghan et al., 2019; Isaksson 2015). Many other species decrease in population size in urban areas or have even disappeared from the urban landscape (Aronson et al., 2014; McKinney, 2002).

Even though we have gathered a lot of knowledge about organismal changes induced by urban environments over the last two to three decades, we still lack a deeper understanding about the factors driving the observed phenotypic (and genotypic) variation between urban and rural individuals, and the physiological

pathways involved. Furthermore, most of the studies conducted so far have assessed the influence of the urban environment by comparing urban versus rural individuals or populations. This dichotomous and comparative approach is fruitful in finding phenotypic variation, but it neglects the relative contributions of different environmental stressors. Especially in the case of urban environments, where pollutants often co-occur (Coté et al., 2016; Ross et al., 2011; Weber, 2009), we must assume that urban stressors rarely act alone and that stressors most often affect multiple traits (Halfwerk and Slabbekoorn, 2015; Munoz and Blumstein, 2012; Swaddle et al., 2015). Important insights into effects of specifics pollutants can be gained by direct manipulation of one stressor during controlled experiments. Studying one stressor at a time will add to our understanding of whether specific stressors contribute to an observed change. Understanding the contribution of different urban stressors to behavioural and physiological changes and which life-history traits are affected, is crucial. For example, from a conservation perspective, it is important to know which stressor is most influential, so that targeted mitigation measures can be applied (Coté et al., 2016; Dominoni et al., 2020a).

Additionally, there is growing evidence that stressor effects are highly context-dependent and that the combined exposure to multiple stressors leads to non-additive effects, meaning that effects are either smaller than the sum of individual effects (i.e., antagonistic interaction) or larger than the sum of individual effects (i.e., synergistic interaction) (Hale et al., 2017; Halfwerk and Slabbekoorn, 2015; Jackson et al., 2016, Piggott et al., 2015). Investigating the interactive effects of urban stressors on life-history traits is of great importance, because gaining a better understanding of the dynamics between stressors will add greater insight into the potential for populations and species to evolve and adapt in the face of environmental change (Todgham and Stillman, 2013). In addition, if conservation measures designed for isolated stressor effects are applied to environments that have co-occurring stressors which might elicit non-additive effects, the measures could be ineffective or even counterproductive (Coté et al., 2016; Dominoni et al., 2020a).

Therefore, to be able to address the biological issues related to urban environments, we need to understand the contributions and effects of single pollutants, but also the interactions between multiple pollutants. This can be best achieved by systematic and robust experimental approaches with full-factorial designs, aiming to disentangle single stressor effects from combined effects and characterising additive, synergistic and antagonistic effects (Schäfer and Piggott, 2018).

Thesis aims

Understanding the relative contributions of each urban stressor to wildlife physiology and behaviour is crucial to be able to address and target specific biological issues related to urbanisation and to counteract future threats. So far, a multi-stressor approach, aimed to disentangle the relative contributions of, and interactions between stressors, has been rarely applied in the context of urban ecology. Hence, the main aims in this thesis were (i) to explore, disentangle, and characterise the single stressor and combined stressor effects of different urban pollutants on avian physiology and behaviour, and (ii) to gain a deeper knowledge of the physiological mechanisms involved in mediating phenotypic responses to the exposure to different anthropogenic pollutants.

I focused specifically on three main urban pollutants: artificial light at night (ALAN), anthropogenic noise and air pollution, especially particulate matter, ozone, and nitric oxides. Additionally, I also investigated the effects of differing diets, vegetation composition and ambient temperature, all of which are affected by human presence. In paper I, I studied behavioural responses to multi-stressor exposure. In papers II–V, I studied the oxidative stress system (papers III, IV and V), immune function (papers II and IV) and plasma fatty acid composition (paper IV and V) as the physiological phenotypic traits which are likely to be key mechanisms of response to human induced environmental change. Extensive knowledge demonstrates clear links between these phenotypic traits and fitness (reproductive success and survival), and hence it is highly likely that these physiological traits could explain observed variation in fitness traits between urban and rural populations of birds. I used both wild (papers II and V) and captive birds (papers I, III and IV) in full-factorial exposure experiments (papers I–IV) and one correlative study (paper V).

Specifically, the questions addressed in the individual papers were:

Paper I: Is the behaviour of birds differently influenced by exposure to the two pollutants ALAN and anthropogenic noise in isolation and combination? Does the combined exposure to two different pollutants have non-additive effects on avian behaviour?

Paper II: Does exposure to ALAN affect baseline innate immune function and innate immune response?

Paper III: Is the oxidative stress status of birds affected by exposure to the three different urban pollutants ALAN, anthropogenic noise, and soot as an air pollutant? Does the combined exposure to three different pollutants have non-additive effects on oxidative stress status?

Paper IV: Is the oxidative stress status of birds affected by different compositions of dietary polyunsaturated fatty acids (PUFAs)? Are different ratios of dietary PUFAS modulating the oxidative stress response to an oxidising air pollutant (ozone) and immune challenge? Do we find non-additive effects of ozone exposure, immune challenge, and differing PUFA diet on the oxidative stress status?

Paper V: How do abiotic (ALAN, air pollution, ambient temperature) and biotic (vegetation composition) characteristics in a bird's territory affect and contribute to nestling oxidative stress status and condition?

In the following section, I will give a brief overview of the biotic and abiotic factors that differ between rural and urban environments and whose impacts were explored in the different studies in this thesis.

Box 1. List of main abriviations used in the thesis

ALAN - artificial light at night

GSH/GSSG - reduced form of glutathione/ oxidised form of glutathione (glutathione disulphide)

LPS – lipopolysaccharide

MDA – malondialdehyde, oxidative damage

OXY – non-enzymatic antioxidant capacity

PBS - phosphate buffered saline

PM - particulate matter

PUFA - polyunsaturated fatty acid

RBC - red blood cells

ROMs - reactive oxygen metabolites, oxidative damage

tGSH – total glutathione

Background

The urban environment

The process of urbanisation is marked by a transformation of natural habitats, leading to a fragmented patchwork of semi-natural habitats and to a landscape consisting of artificial and impermeable surfaces, altered vegetation composition and anthropogenic disturbance (Marzluff and Ewig, 2001; Meyer and Turner, 1992). The increase in human presence and activity is accompanied by a novel set of abiotic and biotic factors. The change, absence or novel presence of these factors can pose challenges to the wildlife living in urbanised areas (Grimm et al., 2008).

Alongside, this transformation of the landscape, other main characteristics that distinguish urbanised areas from rural habitats are different forms of pollution. Most of the pollutants are connected to human activities and transportation networks. Three major pollutants have been identified, acting as stressors in anthropogenic environments, namely air pollutants, anthropogenic noise, and light pollution. These pollutants have also been termed "sensory pollutants", as they affect the perception and functioning of a range of sensory systems (e.g., hearing, vision, olfaction), which animals use to detect environmental information. Though, in the context of this thesis and in avian ecology, air pollution is more linked to pro-oxidant effects on macromolecules (see below), rather than being a sensory pollutant in the common sense affecting, for example olfaction in insects (Leonard et al., 2019).

Below, I will give an overview of the main anthropogenic stressors that characterise urbanised environments and that have been the central themes of the different papers of this thesis.

Abiotic characteristics

Air pollution

Most air pollutants are derived from human traffic-related activities and industry. Incomplete combustion of fossil fuels produces most primary urban air pollutants, such as carbon dioxide (CO_2), carbon monoxide (CO), nitric oxides (NO_x), sulphur

oxides (SO_x), organic volatile compounds (VOCs) and particulate matter (PM) (Hill, 2010). PM is a heterogeneous mixture of solid and liquid particles suspended in the air. The mixture of particles can include metals and polycyclic hydrocarbons, which greatly add to the toxicity of PM (Boström et al., 2002). PM can be divided into different size categories: "coarse" particles categorised as PM_{10}, as they have an aerodynamic diameter of < 10 µm (in comparison a human hair is 50–70 µm in diameter), "fine" particles with a diameter < 2.5 µm as $PM_{2.5}$, and "ultrafine" particles with a diameter < 0.1 µm $PM_{0.1}$ (Hill, 2010). Particles from the size of PM_{10} and smaller can enter airways and are known to cause inflammation in respiratory tissue (reviewed in e.g., Anderson et al., 2012; Auerbach and Hernandez, 2012). Secondary air pollutants are for example formed through reactions of VOCs and nitric oxides, which is the case for ozone (O_3) (Brook et al., 2004; Hill, 2010). Ozone is a highly reactive molecule that has the ability to generate free radicals which target macromolecules (Mustafa, 1990). Emissions from traffic and industry can also contain metals, such as mercury, lead, copper, or iron, which also can act as potent pro-oxidants and toxins (Ercal et al., 2001; Jomova and Valko, 2011). Levels of air pollutants have been decreasing in some areas of the world due to rigid regulations, but there is still a large proportion of the human urban population that is exposed to high and possibly hazardous levels of different air pollutants (European Environment Agency, 2020).

Anthropogenic noise pollution

First, it is important to note that the "natural" environment is not without noise. While natural noise generated by for example wind, water, or other animals can be just as loud as anthropogenic noise (Brumm, 2013). Animals have evolved to, for example, communicate in the presence of such natural noise (Brumm and Zollinger, 2013). The extensive development of transportation infrastructure over the last 50 years has led to an immense increase of noise levels in urban areas (Barber et al., 2009), in addition to an expansion of noise polluted areas, which has diminished areas of quiet habitats. The urban cacophony, produced by cars, trains, airplanes and industrial machinery, is a novel mixture of sounds that animals have to cope with. The spectral composition of urban noise is characterised by intense levels of low-frequency sounds (Slabbekoorn and Peet, 2003) and the acoustic properties of the urban soundscape have been described as resembling a rocky habitat with a lot of sound-reflecting surfaces (Slabbekoorn et al., 2007). In Western Europe, traffic noise exposure has identified by the World Health Organization as the second most important factor of impaired health, only surpassed by fine particulate matter (World Health Organization, 2011).

Artificial light at night (ALAN) pollution

Natural light or photoperiod is one of the most reliable and predictable proximate cues that can be used by living organisms to relay information about the change in the environment into internal daily and seasonal processes (Forster et al., 2004). Organisms have evolved under natural light conditions and are adapted to the daily oscillations of light and darkness. With the extensive use and installation of electrical night lighting during the last century, the loss of darkness has become an increasing concern and ALAN exposure has been recognised as a growing stressor for organisms living in anthropogenic environments (e.g., Davies and Smyth, 2018; Hölker et al., 2010; Richard et al., 2021). While a clear starry night has light intensities of about 0.001 lx and a clear night with a full moon is about 0.1–0.3 lx, ground level illuminance directly under streetlights is between 10 and 40 lx (Gaston et al., 2017, 2013; Longcore and Rich, 2004). A secondary form of light pollution, besides the direct illumination by streetlights, is the so called urban "skyglow". Skyglow is produced when light is reflected or directly emitted upwards and scattered back by molecules in the air, leading to high levels of background sky brightness over large areas (Kyba et al., 2015). It is estimated that about 23% of the world's land surface and even 88% of the European continent experiences light polluted nights (Cinzano et al., 2001; Falchi et al., 2016). While exposure to ALAN in urban areas is a general problem for wildlife, there are other complications accompanying the increased use of night lighting. Since the 1960s, technological advancements have led to a change from narrow spectrum light sources to more broad-spectrum light sources. Unlike incandescent bulbs with a yellow glow and low-pressure sodium lighting with a peak at 589 nm (orange), the recent "white" light sources (e.g., LEDs) are emitting a broad spectrum of wavelengths, with peaks in the blue range (reviewed in Gaston et al., 2012, 2013). This change to light sources emitting more blue and green wavelengths has important implications for how animals respond to artificial light at night (Grubisic et al., 2018; but see McNaughton et al., 2021). Photoreceptors in humans and birds are most sensitive to blue light at wavelengths between 464 and 484 nm, and the receptors should therefore be stimulated to a larger extend by LEDs as light sources (Ouyang et al., 2018; Pauley, 2004).

Ambient air temperature

An additional factor creating a distinct urban climate, besides the main abiotic factors mentioned above, is temperature. Cities are found in general to be 1–4°C warmer than the surrounding non-urban areas, due to a trapping effect of the heat at night and less reflectance of sun rays during the day due to more paved areas (Oke, 2010; Peng et al., 2012; Shochat et al., 2006). This so called "urban heat island" effect has important implications, especially for vegetation phenology (Neil and

Wu, 2006) and thereby also the timing of life-history events for organisms higher up in the food chain (Chamberlain et al., 2009; Jensen et al., 2021).

Biotic characteristic

Vegetation

There are not only changes in the abiotic environment in urbanised areas, but also changes in the biotic environment. Urban vegetation structure and composition is largely different from rural or natural vegetation. For example, non-native plant species are more abundant in urbanised habitats (Aronson et al., 2014). Introduced plant species often differ from native plants in traits like phenology, photochemical composition and suitability as host plants for invertebrates (Cappuccino and Arnason, 2006; Helden et al., 2012; Rodewald et al., 2010). Differences in these traits between native and non-native plant species can therefore have consequences for diversity and abundance of invertebrates (Burghardt and Tallamy, 2015; Jensen et al., 2021; Tallamy et al., 2021; Padovani et al., 2020). From an avian perspective, the phenology and invertebrate abundance of trees is especially important during the breeding season, as insectivorous birds try to match the peak of caterpillar abundance with the peak of nestling food requirements (Both et al., 2009; Narango et al., 2017). Furthermore, traits like phenology can also be influenced by other urban characteristics like the urban heat island effect (see above) (Zhang et al., 2004) or increased levels of ALAN (Dominoni et al., 2020b), which exemplifies the complex interaction network between different stressors. Additionally, urban vegetation (including native plant species) has been found to contain decreased amounts of micronutrients, such as carotenoids, compared to rural vegetation, which has negative impacts throughout the food chain, from leaves to caterpillars to birds (Isaksson, 2009).

Diet and nutrition

Diets of animals living in urbanised areas can differ drastically from diets of individuals in rural or more natural habitats. In urban areas, wildlife has more opportunities to feed on anthropogenic food like litter from garbage bins, or is actively fed by humans either at restaurants, bird feeders, or in parks and gardens (Jones and Reynolds, 2008). These additional sources of anthropogenic food might increase the quantity of food and make feeding opportunities more predictable, but can also create a diet of poorer quality or of different nutritional composition than a natural diet (Andersson et al., 2015; Schoech et al., 2004). For example, important dietary antioxidants and micronutrients can occur in reduced concentrations in the

diets of urban wildlife (Eeva et al., 1998; Isaksson and Andersson, 2008). Poor quality diet can have effects on avian physiology, as for example the composition of fatty acids in great tit (*Parus major*) plasma has been found to differ between urban and rural populations, suggesting differential diets between these environments (Andersson et al., 2015). In this thesis, we focus mainly on fatty acids, more specifically PUFAs, as one of the potential factors creating phenotypic differences between urban and rural populations, if they are for example ingested though food (paper IV), but also as physiological traits (as circulating PUFAs) varying between urban and rural birds (Andersson et al., 2015; Toledo et al., 2016) (paper V). Differential dietary fatty acid composition, and thus membrane composition of different tissues, has been shown to have effects on a wide range of traits, such as thermoregulation, inflammation status, cell membrane fluidity and exercise performance (Ben-Hamo et al., 2011; Calder and Grimble, 2002; Fritsche, 2006; Hazel, 1995; Pierce and McWilliams, 2014). PUFAs contain two or more carbon-carbon double bonds and the most commonly occurring PUFAs are ω6- and ω3-PUFAs, with the "ω" indicating the location of the first carbon-carbon double bond. Among these, linoleic acid (LA, 18:2ω6) and α-linolenic acid (ALA, 18:3ω3) are essential PUFAs, meaning they cannot be produced by the organism itself, but have to be obtained in diet. ω6- and ω3-PUFAs are of great importance to the functioning of many physiological processes, as well brain development and vision (e.g., Newman et al., 2000; Twining et al., 2016a and b; Uauy at al., 2001). Importantly, ω6- and ω3-PUFAs and their metabolites (eicosanoids) have been shown to have opposite effects on inflammatory responses (Larsson et al., 2004; Romieu et al., 2008), with ω6-PUFAs being pro-inflammatory and ω3-PUFAs less or even anti-inflammatory. A diet rich in anthropogenic food sources is likely to have higher ratios of ω6:ω3 PUFAs. For example, sunflower seeds provided at bird feeders contain high amounts of pro-inflammatory ω6-PUFAs, which could have important implications on metabolism, oxidative stress and immune function (Abdulla et al., 2019; Andersson et al., 2018).

Phenotypic traits that respond to urban pollutants

Below I will give an overview of different phenotypic traits that respond to human-induced environmental change and may also mediate downstream variation in additional phenotypic traits between urban and rural populations with subsequent consequences for fitness. This overview is not meant to be comprehensive, but highlights the focus of this thesis. To keep it somewhat short, I will mainly focus on examples from the avian urban ecology literature, but also include examples from other taxa.

Behaviour

Behavioural adjustments are often the first responses to anthropogenic environmental changes. Behavioural adjustments can be used to either reduce or escape exposure to stressors or mitigate their potentially negative effects. Such behavioural changes may be either adaptive and enhance an individual's chances of survival and reproduction, or they may be maladaptive, leading to a lower fitness and potential decline or even extinction of populations. Examples of behavioural adjustments to urban environments are plentiful (Sol et al., 2013; Tuomainen and Candolin, 2011). ALAN and anthropogenic noise are two main pollutants driving behavioural changes in urban habitats. The effects of ALAN exposure on behaviour are found across different taxa (Longcore and Rich, 2004). ALAN mostly affects the natural temporal regimes of light-dark cycles, leading to a disruption or alteration of behaviour (Davies and Smyth, 2018; Gaston et al., 2017, 2013). Large-scale patterns like migration and orientation are often affected by light pollution, since many animals use light cues for orientation. An extreme example of such a disorientating effect is the strong illumination of the September 11 tribute in New York, where van Doren et al. (2017) showed an enormous trapping effect of migrating birds within these light columns. Similar attraction to light and disturbance of movement effects have been shown in moths and other insects (Eisenbeis and Hänel, 2009), mammals (Beier, 2006; Stone et al., 2009), frogs and reptiles (Baker and Richarson, 2006; Perry et al., 2008).

Foraging is another behaviour that has been found to be altered by the exposure to ALAN. Experimentally lit estuaries attracted more small fish and thus generated more optimal foraging conditions for larger predatory fish (Becker et al., 2013). Similarly, shorebirds in lit areas were able to feed for a longer time, enabling a more efficient visual rather than tactile foraging behaviour (Dwyer et al., 2013; Santos et al., 2010). In songbirds, those species that under natural conditions show an early onset of dawn chorus song, show a significant advancement of the singing behaviour when living close to a light source (Kempenaers et al., 2010). Generally, ALAN allows an extension of activities into dark hours, whether that be morning or evening hours (de Jong et al., 2016; Dominoni et al., 2020c, 2014; Stracey et al., 2014). Blackbirds (*Turdus merula),* for example, extend foraging activities into dusk hours in areas with high levels of ALAN (Russ et al., 2015; but see Dominoni et al., 2014). Interestingly, these birds with longer foraging hours did not differ in body condition, indicating that they do not benefit from a prolonged foraging period (Russ et al., 2015). Contrastingly, mice kept under constant light or a bright light/dim light cycle increased body mass through an alteration in metabolism, but without changing food intake or activity (Fonken and Nelson, 2014). Reproductive behaviour of blue tits (*Cyanistes caeruleus)* is influenced by light conditions, with females in territories with ALAN laying eggs earlier and males being twice as successful in obtaining extra-pair paternity (Kempenaers et al., 2010). Circadian disruption through ALAN exposure also manifests itself through disruption of sleep behaviour. Blue tits wake

up earlier at brighter locations (Steinmeyer et al., 2010), and great tits sleep less, spend less time in the nest box, and also wake up earlier, when exposed to ALAN in a nest box (Raap et al., 2015).

Acoustic cues and signals are important tools in animal communication, but also crucial for either predator avoidance or prey detection (Brumm and Zollinger, 2013). Animals are adapted to the soundscape in their natural habitats, but with increased levels of anthropogenic noise, animals face an evolutionarily unprecedented stressor. Effects of anthropogenic noise range from small individual behavioural adjustments to population level responses. These effects may also vary in magnitude, ranging from minor effects such as masking calls of conspecifics to lethal outcomes from acute overexposure (reviewed in Slabbekoorn et al., 2018). Specifically, noise was found to reduce species richness of nesting birds. Surprisingly, the reproductive success of the individuals nesting in noisy areas was higher than compared to quiet areas (Francis et al., 2009). The authors suggest that this is due to a disruption of predator-prey interactions, as the abundance of a main nest predator was negatively influenced by increased noise levels (Francis et al., 2009). Contrastingly, ovenbirds (*Seiurus aurocapilla)* and male reed buntings (*Emberiza schoeniclus)* breeding at noisy sites had lower pairing success (Gross et al., 2010; Habib et al., 2007). These data suggest that noise masks communication calls necessary for pair bonding or mate attraction (see also Swaddle and Page, 2007). There are several options for an animal to overcome the masking effects of urban noise. Birds may either shift frequencies to "predominate" over low frequency urban sound (Gross et al., 2010, Katti and Warren, 2004; Slabbekoorn and Peet, 2003; but see: Zollinger et al., 2017), or birds may shift the timing of their signalling to evade for example noisy rush hours (Fuller et al., 2007; but see da Silva et al., 2014), or increase amplitude (i.e., loudness) of their song (Katti and Warren, 2004; Brumm, 2004).

While it is apparent that both ALAN and anthropogenic noise exposure can have profound effects on behaviour, studies that have investigated the combined effects of ALAN and anthropogenic noise on behaviour are scarce (Hale et al., 2017; Piggott et al 2015; paper I). Some correlational studies found that, for example, zebrafish (*Danio rerio*) changed swimming behaviour when exposed to ALAN and noise separately, but no interactive effects between the two stressors on swimming behaviour were found (Sabet et al., 2016). In birds, nocturnal singing of European robins (*Erithacus rubecola*) was affected by noise levels during the day rather than ALAN levels during the night (Fuller et al., 2007). Moreover, in European blackbirds (*Turdus merula*) and rufous-collared sparrows (*Zonotrichia capensi*s), traffic noise was driving the shift of dawn singing into night hours rather than ALAN exposure (Dorado-Correa et al., 2016; Nordt and Klenke, 2013), though the effects could not be entirely disentangled in the blackbird study (Nordt and Klenke, 2013). Contrastingly, other studies concluded that ALAN is the driving factor behind increased nocturnal activities (da Silva et al., 2014; Dominoni et al., 2013).

Physiology

There are several key physiological mechanisms that potentially mediate the effects of urban stressors into phenotypic variation. Importantly, these physiological mechanisms are not only the molecular mechanistic underpinnings, but also are the phenotypic traits themselves, which are influenced by (anthropogenic) stressors, and differing between urban and rural birds. In this thesis I will mostly treat the physiological traits in focus as phenotypic traits that respond to environmental change, hence adding to phenotypic variation between urban and rural populations. The two main mechanisms I focused on in this thesis are oxidative stress status (Box 2) and immune function (Box 3). Both are known to be shaped by, and respond to environmental changes, and have been shown to differ between urban and rural environments (Isaksson, 2015, 2010). Moreover, adverse effects on both mechanisms have been linked to reduced survival and reproduction (Bize et al., 2008; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Møller and Saino, 2004).

Urban pollutants are expected to increase oxidative stress (Isaksson, 2010) due to their mostly pro-oxidant and pro-inflammatory nature. However, direction and strength of the effects of urban pollutants on oxidative stress physiology are not always predictable (Isaksson, 2020). This might be partly due to the dual nature of the oxidative stress balance (i.e., antioxidant defence system and oxidative damage part), where different studies may find different responses of the different parts of the oxidative stress system. While some studies find for example an increase in oxidative damage in urban-dwelling birds (Amri et al., 2017; Herrera-Dueñas et al., 2017; Isaksson et al., 2009), others find no change in oxidative damage levels (Herrera-Dueñas et al., 2014; Salmón et al., 2018b, 2018a). Similarly, the levels of antioxidant capacity or defence have been found to vary from a decrease of antioxidant components (Amri et al., 2017; Costantini et al., 2014; Giraudeau and McGraw, 2014), no change (Isaksson et al., 2009) or even an up-regulation of the antioxidant defence system (de la Casa-Resino et al., 2015; Rainio et al., 2013) of urban- compared to rural-dwelling birds. Such context-dependency may be driven by either variation in pollutant distribution or concentration, different compositions of pollutants, duration of exposure, availabilities of important nutrients to support metabolism or any combination of the mentioned factors (Isaksson 2020). Studies that have investigated such context-dependency by manipulating specific urban pollutants are scarce but have found that air pollution increased antioxidant capacity (Salmón et al., 2018a, but see Li et al., 2021), noise pollution increased oxidative stress (Injaian et al., 2018), and ALAN-exposure did not affect the oxidative status of great tit nestlings (Raap et al., 2016a; Casasole et al., 2017). Studies that have examined the combined effects of several urban pollutants on avian oxidative stress physiology are nearly absent (but see Casasole et al., 2017; papers III + V).

Box 2: Oxidative stress status

The oxidative stress status is defined by a balance between pro-oxidants and antioxidants (Fig. 1). Oxidative stress occurs if there is an imbalance between reactive oxygen/nitrogen species (ROS/RNS) and antioxidants (Halliwell and Gutteridge, 2015). This imbalance can either be created through low levels of antioxidants or a surplus of ROS and RNS that are, for example, generated in the mitochondria during aerobic metabolism or processes like inflammation, or coming from the environment (Fig. 1; Costantini, 2014). The surplus of ROS and RNS can affect key biological molecules like DNA, proteins, and lipids, which can accumulate irreparable damages, leading to cellular and tissue dysfunction and/or necrosis. The defence mechanism to prevent oxidative damage can be summarised under the term antioxidant system (Surai, 2002). The variety of different antioxidants involved in the scavenging of ROS/RNS is large and their importance and concentrations can differ between tissues as well as between species and life-stages (Birben et al., 2012; Pamplona and Costantini, 2011). On one side, there is the enzymatic antioxidants like, sodium dismutase (SOD; eliminates superoxide radicals), glutathione peroxidase (GP_x; removes hydrogen peroxide and other peroxides by oxidising glutathione), and catalase (CAT; converts hydrogen peroxide to water and O_2), that remove, or transform reactive species into less reactive molecules (Sies, 1997). On the other side, there are non-enzymatic antioxidants, like vitamins, and carotenoids, that need to be extracted from the food. One of the most important intracellular antioxidants is glutathione. It exists in high concentrations in its reduced form GSH and reacts with free radicals to its oxidised form, from which it gets recycled back into GSH by glutathione reductase (Meister and Anderson, 1983).

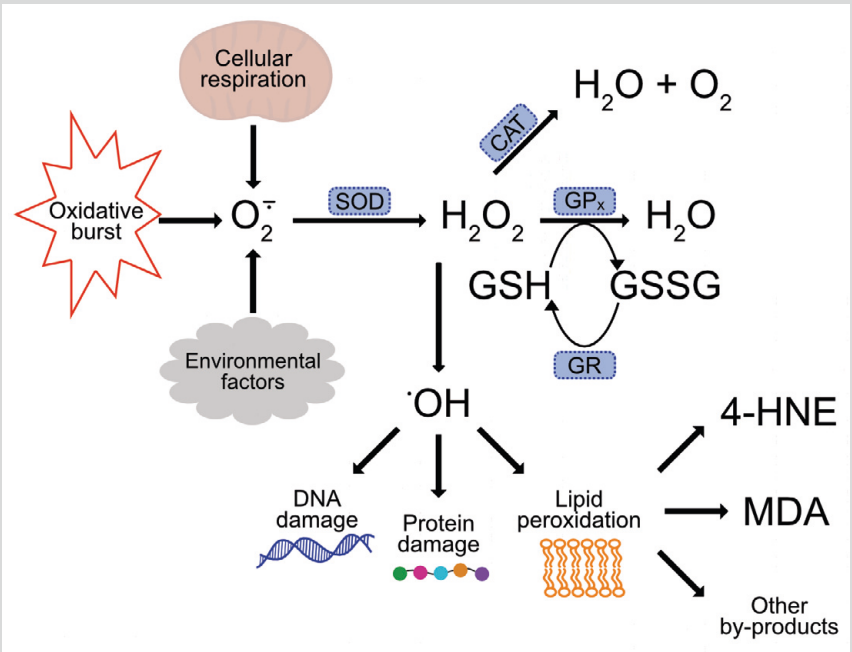

Figure 1. Simplified schematic representation of antioxidant and pro-oxidant interactions. Superoxide anion radicals ($O_2^{\cdot-}$) can by created as a result of cellular respiration, an oxidative burst triggered through an immune challenge, or extrinsic environmental pro-oxidants, such as air pollutants. $O_2^{\cdot-}$ is dismutased by enzymes like the superoxide dismutase (SOD) to hydrogen peroxidase (H_2O_2). H_2O_2 has the ability to penetrate cell membranes, and can therefore spread potential damage. H_2O_2 can be converted via the Fenton reaction, requiring a transition metal, to the highly reactive hydroxyl radical (OH). OH can cause damage to macromolecules like DNA, proteins, and lipids. Lipid damage molecules can be either malondialdehyde (MDA), 4-hydroxynonenals (4-HNE) or others. H_2O_2 can also be can enzymatically removed by catalase (CAT) and glutathione peroxidases (GP_x). The latter requires the reduced form of glutathione (GSH), which gets oxidised to its disulphide (GSSG). GSSG can be recycled back the GSH by the enzyme glutathione reductase (GR). Based on Reiter et al. (2000).

Similar to the oxidative stress system, immune function has been shown to be affected by various factors in the urban environment (Bailly et al., 2016; Ots et al., 1998; Ruiz et al., 2002; but see, Bókony et al., 2012; Ibáñez-Álamo et al., 2020). For instance, some markers of innate immune function have been found to be at lower concentrations after an experimental ALAN-exposure (Raap et al., 2016b, paper II). Moreover, light pollution seems to extend the infectious time period of hosts and increase parasitemia, which might increase transmission risks of zoonotic pathogens (Becker et al., 2020; Kernbach et al., 2019). Contrastingly, Raap et al. (2017) found that noise rather than ALAN altered baseline innate immunity, in this case increasing haptoglobin levels. Much less has been done on the immune responses in relation to urbanisation (Bailly et al., 2016; paper II), compared to the larger number of studies on oxidative stress. Inference from the status of the baseline innate immune function to the capacity to mount an innate immune response is not always linear (Vermeulen et al., 2016), due to possible trade-offs within immune function, but also between the investment in other life-history traits (see below).

Tightly linked to the oxidative stress system and immune function and highly sensitive to environmental changes is the endocrine system. Several responses of endocrine function to the urban stressors have been identified. Main focus has been put on the impacts of urban stressors on the hypothalamic-pituitary-adrenal (HPA) axis, which is involved in regulating and secreting glucocorticoid hormones (e.g., corticosterone in birds). However, studies on responses of the HPA-axis to urban stressors find no consistent patterns, with some studies reporting lower baseline or stress-induced corticosterone levels in urban birds than in rural ones and others finding no differences or increased levels (reviewed in Bonier, 2012; Isaksson and Bonier, 2020). A second important endocrine system is the one involved in creating circadian rhythms. The circadian system is present in nearly all organisms (Dunlap, 1999; Grubisic et al., 2019). Photoreception (in mammals through the retina and in birds additionally through other photoreceptors, for example, in the brain) detects and transforms information of natural dark-light variation into cyclic release of the hormone melatonin mainly from the pineal gland (Dominoni, 2015; Gwinner et al., 1997). Melatonin is commonly known as the “sleep hormone”, as its secretion during dark hours conveys information about the length of dark periods (Gwinner et al., 1997). In the brain, specifically the suprachiasmatic nucleus, melatonin governs synchronisation of the hypothalamic circadian clock, and its peripheral functions are mainly regulation of gene expression, enzymes and ion channels (e.g., Cassone, 2014; Grubisic et al., 2019; Gwinner et al., 1997). Importantly, melatonin has not only chronobiological functions, but it is also considered an important antioxidant and immunomodulator (Moore and Siopes, 2002, 2005; Reiter et al., 2009, 2010; Tan et al., 2010). The photosensitivity of melatonin production makes it susceptible to impacts of light pollution (Grubisic et al., 2019; Ouyang et al., 2015). ALAN exposure has been found to act as a disruptor of circadian rhythms, with animals under increased light conditions showing reduced levels of melatonin (Dominoni et al., 2013; de Jong et al., 2016; paper II). This reduction or disturbance

of melatonin synthesis is one potential mechanism underlying the documented differences in immune function between urban and rural animals (reviewed in Ouyang et al., 2018; paper II).

Physiological processes, such as the oxidative stress system and immune function are thought to be accompanied with certain costs. Whether the currency of this cost is energy or micronutrients that need to be allocated to the maintenance of baseline immune system or to mount an immune response, or as uncontrolled free radicals that inflict oxidative damage, depends on the specific context (Hasselquist and Nilsson, 2012; Isaksson, 2020; Lochmiller and Deerenberg, 2000). In the case of oxidative stress status, for example, a constant investment in the antioxidant machinery and repair mechanisms (e.g., DNA ligase and polymerase) is needed to avoid the negative consequences of high oxidative stress. Similarly, maintaining and using the immune system invokes costs on multiple levels (Hasselquist and Nilsson, 2012). The management and avoidance of costs can lead to trade-offs between competing processes due to a limitation in resources or constraints in physiological or behavioural functions. Thus, since both maintaining oxidative balance and remaining pathogen free are thought to be energetically demanding processes, according to life-history theory, these processes should be competing with other energy-demanding life-history traits like growth, reproduction and other somatic maintenance, such as thermoregulation (Monaghan et al., 2009; Norris and Evans, 2000; Sheldon and Verhulst, 1996).

Box 3: The (avian) immune system

The immune system plays a pivotal role in the body´s defence against attacks from parasites, pathogens and other "non-self" substances. In vertebrates it consists of two interacting branches, the innate and the acquired or adaptive immune system. In this thesis we focused on the innate branch of the immune system, and I will therefore keep the description of the adaptive immune system short. The innate immune system provides an important initial defence of pathogens. It consists of cellular and biochemical defence mechanisms that are constitutively expressed at lower levels, even before an infection happens (i.e., baseline innate immune function) and inducible mechanisms (i.e., acute phase response or innate immune response) that reacts when an invading pathogen is recognised. The innate immune system acts non-specific by recognising well conserved structures on pathogens and responds within minutes after detection of these structures (Abbas et al., 2014; Schmid-Hempel, 2011). The adaptive immune system is a highly specific and specialised immune response towards pathogens. This component of the immune system acquires information about the infection and responds targeted, specifically to the pathogen. As a consequence, the response time is slower (days) than the one of the innate system (Abbas et al., 2014; Schmid-Hempel, 2011).

When invading pathogens are recognised, systemic and metabolic changes are put into motion, a process referred to as the "acute-phase response" (Abbas et al., 2014). Cells involved in the innate immune system are for example monocytes, macrophages, granulocytes and natural killer cells (Abbas et al., 2014). Important non-cellular components that are secreted by, e.g., macrophages, are cytokines, which are soluble proteins that mediate inflammatory reactions and aid the communication between other immune cells (Abbas et al., 2014). Other crucial non-cellular components involved in the acute-phase response are so called acute-phase proteins like haptoglobin. Haptoglobin scavenges free haemoglobin released into the blood by haemolysis, which can act as a pro-oxidant and pro-inflammatory (Matson et al., 2012; Quaye, 2008). The concentrations of acute-phase proteins are increased several folds during an acute-phase response. Importantly, one of the defence mechanisms of the innate immune system against pathogens is the release of reactive oxygen species and nitric oxide (i.e., the oxidative burst) (Hampton et al., 1998). When an immune response is triggered, phagocytes and macrophages will increase the production of superoxide free radicals, which is the start of a cascading production of other free radical species (Costantini and Møller, 2009). ROS are important in killing pathogens, but for chronic inflammation can lead to an increase in oxidative stress with extensive tissue damage.

Material and Methods

Study area

The field work for the studies in papers (II) and (V) was conducted in the southern part of the province of Scania, Sweden (Fig. 2). We used nest box populations in two contrasting habitats, one within the city of Malmö and in a semi-natural recreational nature reserve, Skrylle (Fig. 2). Malmö is the third largest city in Sweden, with approx. 348 000 inhabitants. The urban population was established in 2013 and spans over five different urban parks, with about 400 nest boxes in total. The rural study site was established in 2015 and counts about 390 nest boxes. This study population spans over two main areas that are separated by a road, plus a small number of nest boxes is located about 2km away in a lesser frequented part of an adjacent nature reserve (Dalby norreskog) (Fig. 2). A bicycle path leading through the western part of Skrylle is lit by streetlights during the night. All study populations are thoroughly monitored and followed during the breeding season. The habitat structure of the parks in Malmö is consisting of a mixture of native and non-native trees (paper V), managed grassland and hard surfaces, while Skrylle is deciduous forest.

The laboratory exposure experiments in papers I, III and IV were performed in an exposure chamber of the Aerosol laboratory facilities at the Department of Design Sciences, Lund University (Fig. 4D). The chamber is a stainless-steel room of 21.6 m^3, which can be entered through an antechamber (3.1 m^3), which has air-tight doors. The chamber is under minor positive air pressure. The air supply of the chamber is ensured by an air conditioning system, that provides control over temperature, relative humidity, and air flow. The specific air pollutants (soot in paper III and ozone in paper IV) are generated by additional units situated after an air conditioning and are led into the air stream before entering the chamber. The birds (zebra finches, *Taeniopygia guttata)* that were used for these exposure experiments were kept in outdoor aviaries at the Stensoffa Field Station of Lund University, which is located about 17km east of Lund. The birds were brought indoors for the specific experiments and kept either at Stensoffa in small indoor aviaries (papers I and III) or brought to the Animal facility at Lund University (paper IV) until the exposure to the specific urban pollutants started.

Figure 2: Map of study areas. Lower left map shows parks, with nest boxes in Malmö (red: Rörsjöparken; blue: Kungsparken; green: Slottsparken; orange: Rönneholmsparken; yellow: Pildammsparken) and lower right map shows area of Skrylle nature reserve with nest boxes. For visual reasons, not all nest boxes of our study populations are presented, blue circles represent nest boxes and territories (35 m radius) used in paper V. Dark grey area in top panel marks the general area of Malmö city and Skrylle nature reserve situated in southern Sweden. Adapted from paper V.

Study species

Birds are, in many aspects, an ideal taxon to study the effects of an urbanised environment on physiology and behaviour. They function as sentinel organisms for environmental change, not only in the well-known example of the "canary in the coal mine", but also as a biomonitor of increased exposure to dichlorodiphenyltrichloroethane (DDT), heavy metals and air pollutants (Furness, 1993; Herrera-Dueñas et al., 2014). Birds are thought to be especially sensitive towards increased levels of pollutants due to their higher metabolic rate and more efficient respiratory system compared to mammals (Brown et al., 1997). Below, I will present the two study species on which the results of this thesis are based.

The great tit (*Parus major*, L.1758; papers II and V; Fig. 3A) is a common passerine bird of small size (ca. 18 g body mass and 24 cm wingspan; Robinson, 2005), with a distribution range across much of Eurasia. This species can be found in highly

diverse habitats, ranging from coniferous and deciduous forests, to pastures, suburban gardens, and city parks. Its diet consists mainly of insects during spring (breeding season) and summer and they switch to a more berry- and seed-rich diet during winter and autumn. Great tits are hole-nesting birds and readily use artificial nest boxes when provided. In Southern Sweden, nest building starts in late March, with the first eggs normally being laid around mid-April. Great tits have a clutch size of about 7–9 eggs, which the female incubates for 13–15 days. Nestlings are mainly fed with caterpillars and arachnids and fledge less than three weeks after hatching. The wide distribution, high numbers and use of nest boxes make the great tit an ideal species to study eco-evolutionary processes and eco-physiology in relation to environmental stress. In the context of urban ecology, great tits have been found to show phenotypic differences between urban and rural populations in behaviour, physiology and life-history traits, such as morphology (Biard et al., 2017; Caizergues et al., 2021), cognition (Grunst et al., 2020b; Preiszner et al., 2017), coloration (Biard et al., 2017; Isaksson, 2009), lay or hatch date (Hedblom and Söderström, 2012; Wawrzyniak et al., 2015), clutch size (Hõrak, 1993), oxidative stress physiology (Isaksson et al., 2005; Isaksson et al., 2017) and immune function (Bailly et al., 2016). Additionally, genotypic differences have been found comparing transcriptomes and gene expression between urban and rural tits (Perrier et al., 2017; Salmón et al., 2021; Watson et al., 2017).

Figure 3. Study species used in this thesis. A) Great tit (*Parus major*); papers II and V. B) Zebra finch (*Taeniopygia guttata*); papers I, III and IV. Photo A) by Frank Vasser and B) by David Cook, both licenced under CC-NY 2.0.

The zebra finch (Reichenbach 1862; papers I, III and IV; Fig. 3B), is a small passerine (ca. 14 g, wingspan of about 22 cm), occurring on the Australian mainland, where it is the most common and widespread of the Australian grassfinches. Zebra finches are primarily granivorous and inhabit mainly dry wooded grassland. They breed in loose colonies of about 50 nests and gather year around in social flocks of 100 or more birds. The zebra finch was first used as a study species for species-specific ornamentation in the 1950s and has since evolved into one of the main avian model organisms for laboratory studies (Griffith and Buchanan, 2010). Their social behaviour in flocks, domestication, and fast accumulation of knowledge ranging from behaviour to physiology and genomics makes the zebra finch an ideal species for the laboratory experiments of this thesis.

Field experiments

As *artificial light at night (ALAN)* is one of the most abundant anthropogenic pollutants nowadays, we were interested in investigating the effects of exposure to ALAN on great tit nestling physiology. The study was conducted in "naive" nest boxes in the Skrylle nature reserve forest that were prior not exposed ALAN. We installed a small LED lamp inside the nest box on day 7 after hatching (Fig. 4A). The LED emitted 3 lx of warm white light (2700 K – 3000 K) at the height of the nestlings and was left on constantly until day 14. All experimental nest boxes were installed with an ALAN set-up, but for only half of the nest boxes we switched the lights on. (Paper II)

In the papers II and IV, we wanted to know how the *immune response* is altered after exposure to ALAN in great tit nestlings and whether the additional challenge of mounting an immune response alters the oxidative stress status of zebra finches, respectively. In both experiments we focused on the innate branch of the immune system, which is, after the physical barrier of the skin, the first line of defence against invading pathogens. We triggered an innate immune response by subcutaneously injecting a dose of lipopolysaccharide (LPS). LPS is an endotoxin found in cell walls of Gram-negative bacteria, which elicits an acute-phase immune response, resulting in an inflammatory reaction (Owen-Ashley and Wingfield, 2007). The magnitude of this immune response can be measured already a few hours after the challenge using several immune parameters (see below).

To gain a better understanding of the impacts of *abiotic and biotic characteristics in territories* of great tits breeding in Malmö and Skrylle, we performed a thorough mapping of the territory (35 m radius) around 29 nest boxes that were used in paper V (Fig. 2). Within this circle area we quantified biotic and abiotic parameters that are known to influence bird breeding performance and health. As biotic factors we counted the total number of trees, the number of oak, birch, beech and non-native

trees, as well as the total number of tree species. As abiotic factors we used the number of streetlights within the monitored territories as a proxy for light pollution and we assessed the air quality by measuring the concentrations of particulate matter ($PM_{2.5}$) and nitrogen dioxide (NO_2). Air quality was measured by installing a PM-sensor and a NO_2-diffusion sampler about 1.5 m above the nest box (Fig. 4B and C), starting on day 7 (for NO_2) and day 11 (for PM-sensor) after hatching, respectively. The air quality measurements lasted until day 14.

Laboratory experiments

For the papers I, III, and IV, we manipulated levels of *ALAN, anthropogenic noise and air pollution*. More specifically, in paper I we were interested in how light and noise pollution interacted to affect behaviour. In papers III and IV, we added air pollution (paper III soot, paper IV ozone) to the other two urban pollutants and studied their interactive effects on physiology. We simulated urban light pollution by placing a LED lamp inside the exposure chamber that emitted light at an intensity of about 13 lx. The ALAN was on during the dark hours of the experiment. Noise pollution was simulated by playing a recording of traffic noise from a busy road in Copenhagen, Denmark, at 70 dBA during the daytime hours of the experiment and at 45 dBA during the dark hours. In the experiment for paper III, we used elevated levels of soot ($PM_{2.5}$; weekly concentration of 100 $\mu g\ m^{-3}$) as an air pollutant, while we used elevated levels of ozone (109 ppb) as an air pollutant in the experiment for paper IV. The exposure to all the pollutants lasted for five days.

Differences in *diet* are known to have consequences for physiological traits and immune function, and urban and rural diets have been found to differ in quantity and quality (Andersson et al., 2015). One aim of the experiment in paper IV was to investigate whether differences in urban and rural diets affect the oxidative status of birds. We simulated urban- and rural -like diets by increasing the ω6-PUFA and ω3-PUFA content in their daily diets, respectively. For the former, we mixed sunflower seed oil and coconut oil into the seed mixture in a ratio of 1:33.3, which the birds normally receive. Similarly, we mixed algae oil into the seeds to achieve an increase in ω3-PUFAs. Birds were put onto the treatment specific diet six days before the air treatment started and ingested the specific diets for 11 days in total.

We recorded the behaviour of the zebra finches for the experiment of paper I by mounting video cameras on each of the experimental cages in the exposure chamber. The focal behaviours analysed in the video clips were feeding, resting, preening and movement. We used video clips recorded on three of the five experimental exposure days, with three clips (morning, noon and afternoon) per day and per cage. The proportion of birds per cage displaying the specific focal behaviour per 10-minute video clip was recorded, using a 10 s snap-shot sampling technique.

Figure 4. Representative pictures of different methods used. A) LED lamp emitting 3 lx, installed in a nest box of great tits. Red arrow indicates position of light source; paper II. B) NO_2-diffusion sampler and PM-sensor installed on tree above nest box; paper V. C) Close-up of PM-sensor used in paper V. D) Picture inside exposure chamber used in papers I, III and IV.

Laboratory analysis

Oxidative stress status biomarkers

We measured several aspects of the oxidative stress status, i.e., oxidative defence mechanisms and parts oxidative damage, in order to gain a more complete picture of the balance between defence and damage. We used blood as the tissue of choice, because of the minimal invasive sampling and the possibility of longitudinal sampling. We followed well established protocols previously used in other avian studies (see details below).

To quantify the oxidative defence we measured the total concentration of glutathione (tGSH; papers III and IV) and the levels of its oxidised form of glutathione (GSSG) in red blood cells (RBC) (paper IV), enabling us to calculate the ratio between the reduced and the oxidised form of glutathione (GSH/GSSG). We followed protocols adapted for microplate readers (Baker et al., 1990; Isaksson et al., 2013) Additionally, we measured the plasma non-enzymatic antioxidant capacity, using the OXY-adsorbent assay (Diacron, Italy; papers III and IV; Costantini et al., 2006) and the ferric reducing antioxidant power (FRAP) assay (paper V; Benzie and Strain, 1996). The latter is known to be influenced by uric acid levels (Cohen et al., 2007). In order to correct the FRAP values, we quantified the concentration of uric acid, using a commercial kit (SPINREACT, Sant Esteve d´en Bas, Spain) based on the uricase/peroxidase method, following Salmón et al. (2018a). The concentration of oxidative damage was measured using the dROM assay (Diacron, Italy; papers III and IV; Costantini et al., 2006) and the amount of malondialdehyde (MDA) was quantified in paper (V) as a biomarker for lipid peroxidation, following the protocol in Andersson et al. (2018). All assays apart from MDA used absorbance measures using a microplate reader (FLUOstar OMEGA, BMG LABTECH) and MDA was quantified using Gas Chromatography/Mass Spectrometry (GC-MS).

Immune function biomarkers

Haptoglobin and nitric oxide

We utilized two biomarkers to quantify the baseline of the innate immune system and the magnitude of an acute-phase innate immune response (paper II). Specifically, we quantified the concentration of haptoglobin in the plasma, which is an acute-phase protein that binds free haemoglobin, which would otherwise be able to act as a pro-oxidant (Matson et al., 2012; Quaye et al., 2008). Hence, levels of haptoglobin are expected to increase following an immune challenge (Matson et al., 2012). We followed previously published protocols (Hegemann et al., 2012; Matson

et al., 2012). As a second biomarker, we measured the amount of nitric oxide in plasma, which is a multifunctional signalling molecule and is produced by activated macrophages and other immune cells to kill pathogens (Bogdan, 2001; Coleman, 2001; Sild and Hõrak, 2009). For this assay we followed the protocol previously described in Sild and Hõrak (2009). Both assays are spectrophotometric assays, using microplates.

Fatty acids

Fatty acid composition in great tit nestling plasma (paper V) and in a sub-sample of zebra finch females (paper IV) was quantified to assess the effect of abiotic and biotic factors (paper V) and confirm the diet treatment was casing the intended change in circulating FAs in paper (IV). We performed a total lipid extraction, using a fatty acidmethyl esters (FAME) assay and quantified fatty acid profiles by Gas Chromatography/Mass Spectrometry (GC/MS), following the protocol published in Andersson et al. (2018). From these profiles we calculated the relative abundance of certain fatty acids, an unsaturation index, the relative proportion of PUFAs and the ω6:ω3 PUFA ratio.

Melatonin

To assess if our ALAN exposure treatment in paper II caused a change in circulating melatonin, we quantified plasma melatonin levels of great tit nestlings, by performing radioimmunoassay following chloroform extraction. Details of this assay have been published in Fusani and Gwinner (2004) and Goymann et al. (2008).

Characterisation of interactions

In papers I, III, and IV, we characterised significant interactions following Piggott et al. (2015) and Hale et al. (2017). This classification system is based on additive null models and combines and compares effect size and direction of the single stressor effects (compared to control group; A, B or C in Fig. 5), estimated additive effect (A + B or A + C; Fig. 5) and predicted interaction effect (statistically significant non-additive effect; A x B or A x C in Fig. 5) (Hale et al., 2017; Piggott et al., 2015; Fig. 5). We used estimated marginal means (aka least-square means; Searle et al., 1980), calculated from the post-hoc pairwise comparisons (relative to the control) from the final linear mixed models, using the R package "emmeans" (Lenth et al., 2020). To estimate the additive effects, we summed the parameter effects of the two main effects (relative to the control). The direction of the main single stressor effects could go either in the same direction (negative or positive; left/blue panel in Fig. 5) or in the opposite direction (right/green panel in Fig. 5). We considered the combined effect of two stressors (interaction term) as synergistic

if the effect size was larger (positive or negative) than the effect size of the estimated additive effect (Fig. 5). Similarly, we considered an interaction to be antagonistic if the effect size of the interaction was smaller than the estimated additive effect (Fig. 5). For details see Piggott et al. (2015).

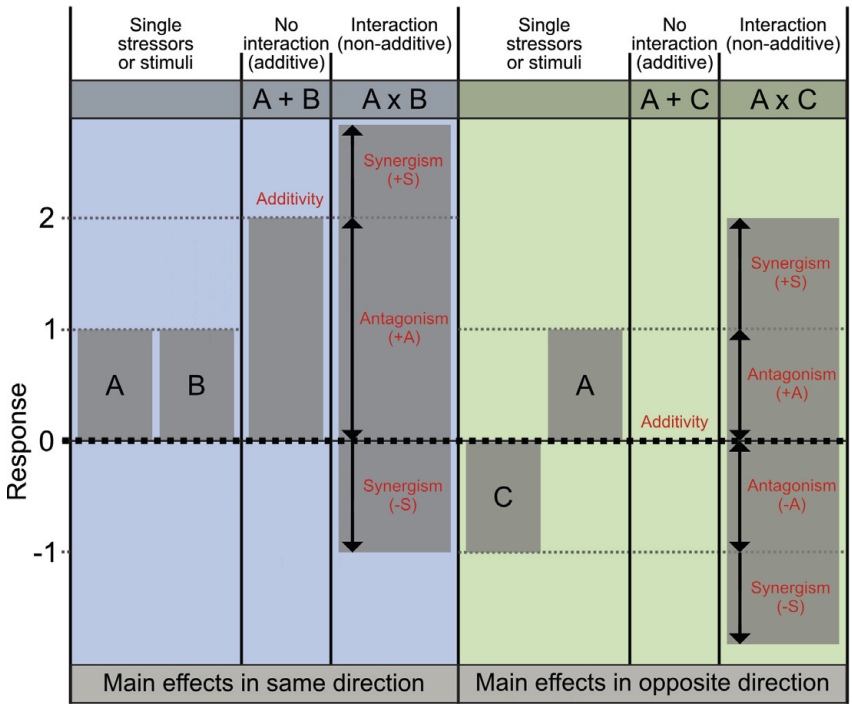

Figure 5. Classification of interactive effects between two stressors or stimuli (A × B and A × C) based on direction and size of main and interactive effects. Left side (blue) shows potential responses to two stressors A and B, when both have significant responses in the same direction. Right side (green) show potential responses to two stressors A and C, when both have significant effects in opposing directions. Grey bars show main effects of single stressors in comparison to control (zero line), expected additive value of responses (sum of single main effects), and interactive effects. Red text and range of arrows describe classification of interactions following Piggott et al. (2015). Synergistic interactions are larger than expected from the additive effect. Positive synergy (+S) is more positive than the expected additive effect and negative synergy (-S) is more negative than the expected additive effect, depending on direction of the individual effects. Antagonistic interaction effects are smaller than expected from the additive effect. Modified from Hale et al. (2017).

Results and Discussion

The different abiotic and biotic stressors found in urbanised areas (reviewed above) pose novel challenges to wildlife. Such challenges can be met in different ways, but the behavioural and physiological mechanisms of responses to urban stressors remain still poorly understood. Likewise, to a large extent, we lack knowledge of how different stressors interact and what the responses are to such combined exposures to multiple stressors. On the one hand, a stressor can act on several sensory modalities and thus elicit a multitude of different responses (e.g., Halfwerk and Slabbekoorn, 2015). On the other hand, physiological mechanisms are highly integrated, meaning that most physiological systems never act alone, but they affect each other's outcomes and/or have the possibility to act through different pathways, albeit leading to the same outcome (Costantini et al., 2011; Isaksson et al., 2011; Salmón et al., 2021). Such a strong integration or robustness of systems might be the reason for sometimes conflicting results and high context-dependency of responses that have been found in this thesis research area.

Behavioural effects

ALAN and anthropogenic noise as modulators of behaviour (Paper I)

In *paper I,* we investigated the effects of exposure to ALAN and anthropogenic noise on different behaviours. These two urban pollutants have received great interest, as they have been shown to have profound impacts on animal behaviour (see above). We used a full-factorial experimental design to be able to disentangle relative effects of these pollutants on different behaviours.

We found that the two stressors mostly affected different behaviours independently, except for a non-additive effect on resting behaviour (Fig. 6A–D). In detail, our results showed that noise exposure decreased the proportion of birds feeding by 27.6% (Fig. 6D) compared to non-exposed birds. The reduction of feeding behaviour by noise exposure may stem from an increase in vigilance behaviour due to the masking effect that increased noise levels can have on, for example, predator cues. Indeed, studies in birds have shown an increase in vigilance behaviour and a decrease in foraging during periods of loud noise (Klett-Mingo et al., 2016; Quinn

et al., 2006). Such a change in behaviour may indicate, on the one hand, a perturbating effect of an anthropogenic pollutant on a sensory modality (e.g., hearing) or, on the other hand, a change of information acquisition from one sensory modality to another (e.g., vision). Importantly, depending when - in respect of the annual cycle - noise pollution is affecting individuals, it may also have negative impacts on reproductive success. Injaian et al. (2018) exposed tree swallows (*Tachycineta bicolor*) to traffic noise during chick rearing, which led to smaller and later-fledging nestlings, but no effect on fledging success was found.

Although we found that noise exposure reduced the proportion of birds found feeding, we did not detect an effect of noise exposure on body mass at the end of the experiment. Birds that were exposed to ALAN were heavier after the experiment than birds without ALAN exposure. Unfortunately, we do not have data on the behaviour during the night, but an increase in body mass in ALAN-exposed birds suggests that ALAN allows an extension of foraging into the normally "dark hours", which may lead to a higher caloric uptake and hence heavier individuals. The extension of perceived daytime by ALAN, and hence possibilities to forage, has been shown in other avian studies (de Jong et al., 2016; Dominoni et al., 2020c, 2014; Stracey et al., 2014; see discussion in General introduction for more details).

Additionally, exposure to ALAN decreased the proportion of birds found moving during the day by 27.4% compared to non-ALAN-exposed birds (Fig. 6A). In line with this, the combined exposure to ALAN and noise led to an antagonistic effect (less than expected from the additive sum of the single effects) on resting behaviour, where more birds that were exposed to only ALAN or only noise were found resting than non-exposed birds, but the combined effect of both stressors was on a similar level as the ALAN exposure effect alone (Fig. 6C). The interaction effect is likely driven by ALAN (see also Dominoni et al., 2020c), as photoperiod is a strong entrainer and synchroniser of behavioural and physiological processes (Cassone, 2014; Gwinner et al., 1997). Hence, an overriding effect of ALAN over noise may not be a surprise.

As we performed this experiment on captive zebra finches and under highly controlled conditions, a generalisation onto natural environments is limited. Whether, for example, a reduction of 27% of feeding behaviour due to noise exposure has fitness and survival consequences in a natural setting can only be speculated. However, one can assume that a reduced caloric intake may have effects on growth, body maintenance, reproductive success and/or survival. Especially in the wild, where conditions are often harsher than in captivity, with unpredictable food sources and variable weather conditions, one might expect higher costs of a reduction in foraging than in a laboratory setting. Similarly, a reduction of movement behaviour during the day due to ALAN exposure may have profound consequences on survival, as birds could be less reactive towards predators.

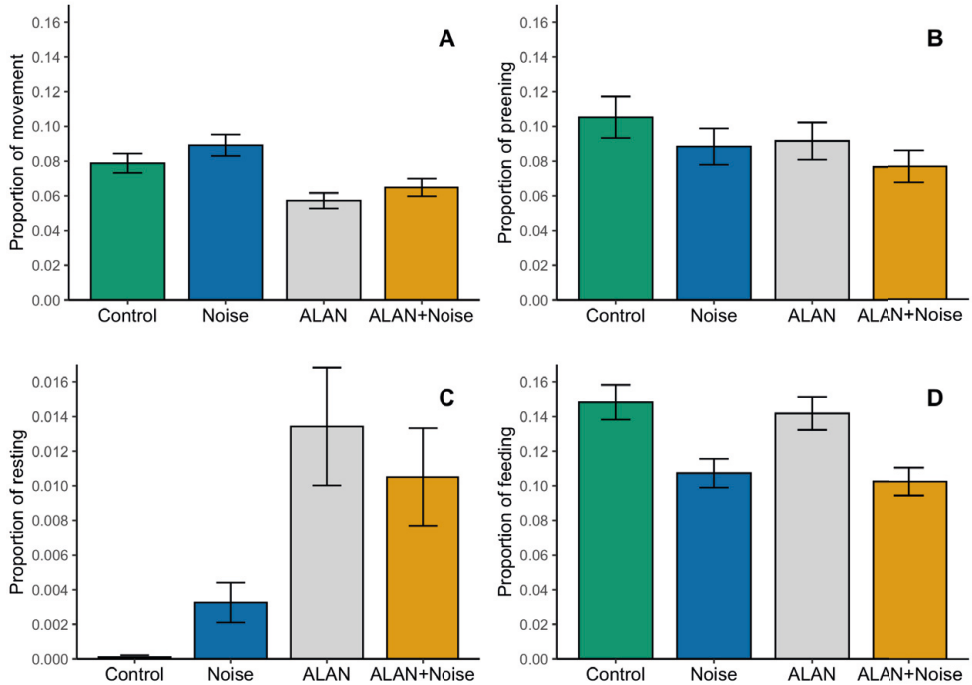

Figure 6. Effects on proportion of movement (A), preening (B), resting (C) and feeding (D) behaviour of single exposure to artificial light at night (ALAN; grey) and anthropogenic noise (blue) and the combined exposure to ALAN and noise (yellow) and non-exposed birds functioned as control group (green). Note the different scale for (C). Estimated marginal means ± standard errors calculated from final models are presented. Details in paper I.

Physiological effects

ALAN exposure alters immune function (Paper II)

The results in *paper I* indicated that ALAN is a strong driver of behavioural changes. In *paper II,* we investigated the physiological changes induced by exposure to ALAN. As discussed above, photoperiod entrains and synchronises physiological processes. The disrupting effect of ALAN exposure on melatonin production can have extensive effects on other physiological processes. The hormone melatonin has multiple biological functions, ranging from the well-known circadian regulation and mediator of day length to being an important antioxidant and immunomodulator (see above). Hence, ALAN-induced changes in melatonin levels may have consequences on immune function. We explored this question in *paper II* by exposing seven-day old great tit nestlings to five days of ALAN of 3 lx, and

subjecting them to an immune challenge (with LPS) that elicited an innate immune response (see Box 2).

As expected, we found that ALAN exposure reduced melatonin levels. In our case, ALAN-exposed nestlings had only about half the concentration of circulating melatonin compared to dark-night nestlings. This indicates that ALAN levels as low as 3 lx are sufficient to function as endocrine disruptors. Previous studies reported similar melatonin-reducing effects of ALAN in adult birds, however the response seems to be dose-dependent (Dominoni et al., 2013; de Jong et al., 2016). The fact that we find a significant reduction of melatonin concentrations due to ALAN exposure as early as post-natal development, in birds, may have important implications for developmental trajectories and later-life consequences (Zeman and Gwinner, 1993; Zeman and Herichová, 2011).

We found that ALAN exposure decreased baseline levels of nitric oxide, but not levels of haptoglobin. This is partly in contrast to a previous finding in ALAN-exposed great tit nestlings, where a two-day ALAN exposure decreased baseline nitric oxide levels, but increased baseline haptoglobin levels (Raap et al., 2016b). Overall, these results confirm that exposure to ALAN has the capacity to alter indices of baseline innate immune function.

The immune challenge showed that ALAN-exposed birds respond differently compared to dark-night birds, with ALAN birds showing a smaller change in the two measured biomarkers than dark-night birds (Fig. 7A and B). These findings indicate a potential impairment of the capacity to mount an innate immune response due to ALAN exposure. In detail, haptoglobin levels after the immune challenge were marginally lower in the ALAN birds, compared to the dark-night birds (Fig. 7B). Similarly, ALAN birds had a weaker innate immune response than dark-night nestlings in terms of nitric oxide levels, but unexpectedly, nitric oxide levels were lower post-immune challenge than pre-challenge (Fig. 7A). Nitric oxide production is considered to be inducible by an activation of the immune system and thus expected to present higher values following an immune challenge (Coleman, 2001; Wink et al., 2011). Whether we have missed the peak of nitric oxide production by sampling 15.5 hrs after the challenge or if other reasons are behind these findings requires further investigation.

We did not find an effect of ALAN exposure on growth of nestlings from day 7 to day 13. This indicates that lower levels of melatonin were not sufficiently low to affect body mass gain, which is an important indicator of fledgling survival (Monrós et al., 2002). A preserved growth rate irrespective of ALAN exposure and an altered immune response may also indicate a potential trade-off decision between these two life-history traits.

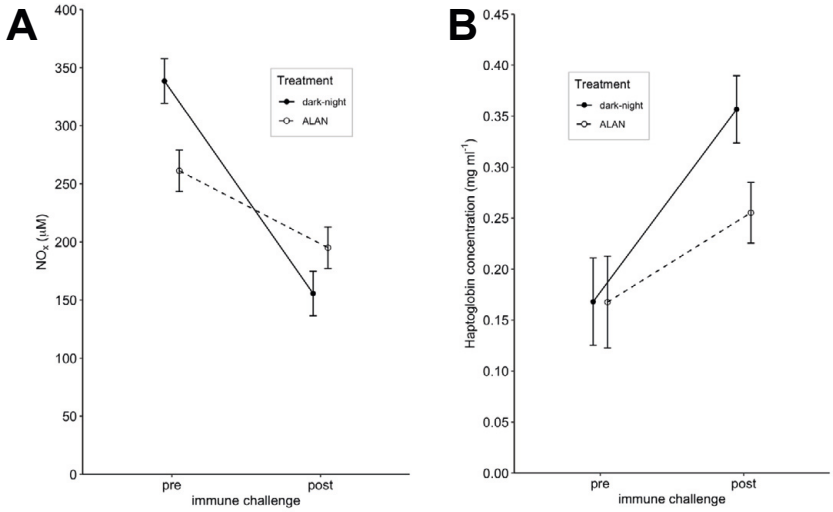

Figure 7. Effect of exposure to artificial light at night (ALAN) on nitric oxide (NO_x) concentrations (A) and haptoglobin concentration (B), following immune challenge, in great tit nestlings. Closed circles represent dark-night and open circles ALAN-exposed nestlings. Estimated marginal means ± standard errors calculated from final model are shown. Details in paper II; Ziegler et al. (2021).

Effects of single and combined exposure to ALAN, anthropogenic noise, and particulate matter on oxidative stress physiology (Paper III)

In *paper III*, we were interested in the effects of multiple stressors on oxidative stress status, when acting in combination and alone. Manipulating exposure levels of several urban pollutants in the wild, while controlling for other confounding factors, is notoriously difficult. Hence, we utilised a controlled laboratory experiment in order to disentangle the relative effects of each of the manipulated pollutants. In detail, we exposed captive zebra finches for five days in a full-factorial design to elevated levels of ALAN (about 13 lx), anthropogenic noise (daytime levels about 70 dBA and nighttime levels about 45 dBA, as measured without birds in the exposure chamber), and/or air pollution in the form of soot particles ($PM_{2.5}$ 24h-average: 100 µg m^{-3}). We measured different markers of the oxidative stress system before the pollutant exposure and at the end of the exposure.

Our results showed that, indeed, combined exposure to two sensory pollutants can lead to unexpected non-additive effects. In detail, we found that the combined exposure to ALAN and noise increased levels of the antioxidant, total glutathione, more than the additive effect from the two single pollutant effects would have estimated (positive synergistic effect). Additionally, the combined exposure to ALAN and noise also affected the levels of oxidative damage in a non-additive manner. We found that the exposure to either ALAN or noise did not have an effect on oxidative damage levels compared to birds not exposed to ALAN and noise, but

the combined exposure to ALAN and noise decreased oxidative damage compared to noise-exposed birds. Furthermore, this interaction can be classified as a negative synergistic effect, meaning that the direction of the combined effect is opposite to the direction of the single exposure effects and expected additive effect. More intuitively though, one can term this pattern also a reversal effect, since from a more biological point of view (rather than statistical), low levels of oxidative damage can be seen as positive for overall physiological health. Our results stand partly in contrast with other studies investigating interactive effects between two pollutants. While, for example, a correlative study in wild great tit nestlings found no effect of different levels of ALAN and/or noise on the oxidative stress status (Casasole et al., 2017), another experimental exposure study in great tit nestlings found that nestlings have higher levels of an innate immune biomarker when exposed to noise, but not to ALAN or the combined exposure (Raap et al., 2017). Deviations in findings may either arise from species-specific responses (Salmón et al., 2018a, Wilson et al., 2021), different responses due to different life-stages (Isaksson, 2010) or differences in stressor intensities and exposure durations (Isaksson, 2020).

Even though the levels of tGSH and the oxidative damage levels were not significantly correlated within an individual, our results suggest that exposure to the combination of ALAN and noise incurs lower oxidative stress than exposure to one of the pollutants on its own (Fig. 8A and B). An exposure to multiple stressors might trigger an up-regulation of antioxidant defences either in expectation of an increase in ROS levels or as a result of increased ROS levels, which subsequently leads to increased scavenging of ROS and lower levels of oxidative damage to macromolecules. Such an up-regulation of the antioxidant machinery under stressful conditions has been documented before (e.g., Herrera-Dueñas et al., 2017; Salmón et al., 2018b; Tkachenko and Kurhaluk, 2013; Watson et al., 2017). Whether such an up-regulation of the antioxidant defence comes with any costs has to be determined in further experiments. Especially in the wild, where resource limitation is more acute, potential trade-offs with other physiological processes, such as immune function, are possible, as well as long-term trade-offs between the oxidative stress system and life-history traits have been put forward (Monaghan et al., 2009).

Surprisingly, we did not find any effects of soot exposure alone or in combination with other pollutants on oxidative stress physiology, even though experimental exposure of, for example, humans to particulate matter is strongly associated with negative health impacts (e.g., Anderson et al., 2012; European Environment Agency, 2020). We can give several speculative explanations for our non-significant results involving soot exposure. First, we generated rather "clean" soot (i.e., black carbon without additional metals or organic attachments), which might not elicit strong adverse health effects. Second, soot impacts might have happened more locally (Sanderfoot and Holloway, 2017), such as in the lung tissue, while impacts of ALAN and noise exposure are likely to be systemic. Hence, our measurement of oxidative stress status in blood might not have captured local

inflammation processes in the lung tissue (but see Margaritelis et al., 2015). In line with the previous explanation, in this experiment, the definition of sensory pollutant applies more to the two pollutants ALAN and noise, than it does to the applied air pollution. This is because, here, we examine the potential pro-oxidant effects of soot directly on macromolecules, rather than treating soot as a stressor for a sensory system like olfaction, which has been shown to be negatively affected by air pollution in insects for example (Lusebrink et al., 2015; Girling et al., 2013; Reitmayer et al., 2019).

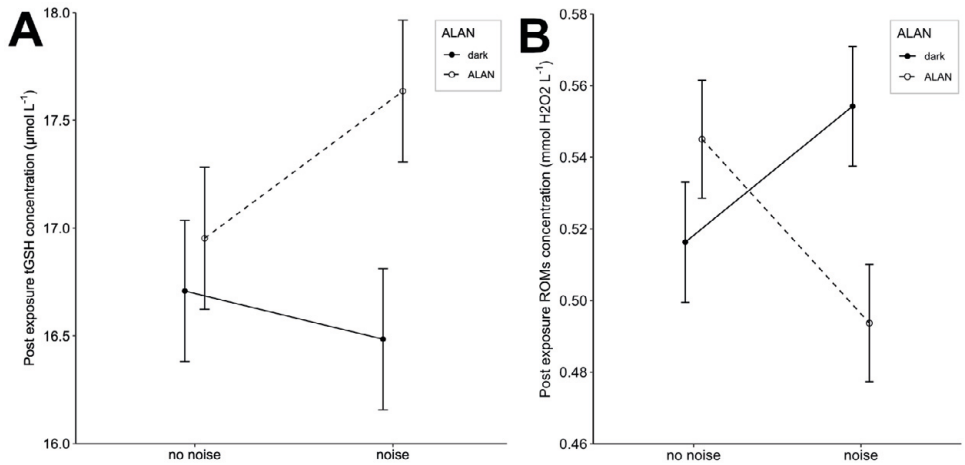

Figure 8. Effects of exposure to artificial light at night (ALAN; dark/ALAN) and anthropogenic noise (no noise/noise) on post-exposure concentration of total glutathione (tGSH) in red blood cells (A) and post-exposure concentration of reactive oxygen metabolites (ROMs) in plasma (B) of zebra finches. Estimated marginal means ± standard errors calculated from final models are shown. Details in paper III.

Different dietary ω6:ω3 PUFA ratios as modulators of oxidative stress responses to ozone and immune challenge (Paper IV)

In this paper, we investigated whether differential diets modulate the physiological response towards an oxidising air pollutant and an immune challenge. To that end, we fed captive zebra finches a diet differing in ω6:ω3 PUFA ratios, where one group received a diet rich in ω6-PUFAs and one group was fed a diet rich in ω3-PUFAs. Half of the birds of each diet treatment were then exposed for five days to either increased levels of ozone (about 109 ppb) or ambient filtered air, while continuing to receive their respective PUFA diet. On the morning of the fifth day, we subjected half of the birds of each air treatment group to an immune challenge, by subcutaneously injecting LPS, while the other half received a control injection of

phosphate buffered saline (PBS). About 11 hours later, at the end of the fifth ozone/ambient air treatment day, we took a blood sample, from which we measured the oxidative stress status of the birds.

We found that diet had an effect on the antioxidant capacity of the birds (Fig. 9B), but not on tGSH. In detail, an ω3-rich diet led to a lower plasma non-enzymatic antioxidant capacity (OXY) than a ω6-rich diet, irrespective of air treatment (Fig. 9B). Conversely, ozone/ambient air treatment had an effect on tGSH levels, where exposure to increased levels of ozone led to lower concentrations of tGSH, while the non-enzymatic antioxidant capacity was not affected by high ozone levels. Previous studies on effects of ozone and general air pollution on oxidative stress status found conflicting results (Avissar et al., 2000; Kadiiska et al., 2011; Kodavanti et al., 1995; Li et al., 2021; North et al., 2017; Rietjens et al., 1985; Salmón et al., 2018a), indicating high context-dependency (Isaksson, 2020). Additionally, we found that levels of oxidative damage (ROMs) were not affected by either of the diets or the ozone/ambient air treatment, but the immune challenge (LPS injection) increased ROMs concentration compared to PBS-injected birds. Increased oxidative damage following an immune challenge has been shown previously (e.g., Armour et al., 2020; Cray et al., 2009; van de Crommenacker et al., 2010) and is likely the result of an "oxidative burst" triggered by the immune activation to fight invading pathogens (Schmid-Hemple, 2011).

Furthermore, we found that, indeed, different ω6:ω3 PUFA ratios are able to modulate the oxidative stress response towards a pro-oxidant challenge at least in one marker of oxidative stress status (Fig. 9A). An interactive effect between diet treatment and ozone/ambient air treatment on the GSH/GSSG ratio indicated that birds fed a ω3-rich diet had a lower GSH/GSSG ratio under clean ambient air conditions than birds fed a ω6-rich diet, but this effect disappeared in the birds that were exposed to elevated ozone levels (Fig. 9A). This ratio serves as an index of current oxidative stress, with a lower ratio indicating higher current oxidative stress and *vice versa*. This suggests, on the one hand, that birds on a ω3-rich diet experience higher current oxidative stress when in clean ambient air conditions than birds on a ω6-rich diet. In combination with the finding of a lower antioxidant capacity in ω3-rich diet birds, one may conclude that increased dietary levels of ω3-PUFAs negatively impact the oxidative stress status of birds. There is for example an ongoing discussion whether ω3-PUFAs may act under certain circumstances act as pro-oxidants, such as for example high dosage, and due to their high susceptibility to peroxidation because of a high double bond number (Corteselli et al., 2020; Serini et al., 2011). Similar arguments have been made for carotenoids (important dietary antioxidants) in high doses (Giraudeau et al., 2016; Huggins et al., 2010; Palozza, 1998).

On the other hand, one could argue that birds on a ω3-rich diet maintain a greater capacity to react to an oxidative challenge, as the difference between the ω3-rich diet groups was larger than the one between the ω6-rich diet groups. Supporting this argument, it has been suggested that glutathione levels are homeostatically regulated, meaning that cellular environments with an increased ROS production, such as mitochondria, or that are under a current oxidative challenge, have increased GSH levels to meet the required redox state (Smith et al., 1996; Schafer and Buettner, 2001). Hence, a high ratio of GSH/GSSG and higher levels of tGSH in ω6-fed birds compared to ω3-fed birds may suggest a "precaution" taken in order to be able to counteract potential negative impacts of ω6-derived pro-inflammatory metabolites. As this experiment was performed with captive birds under *ad libitum* food conditions and without other potential harsh conditions found in the wild, we think that an up-regulation of the antioxidant machinery is a likely scenario. The fact that we did not find a dietary effect on oxidative damage levels suggests though that both strategies suggested above are possible.

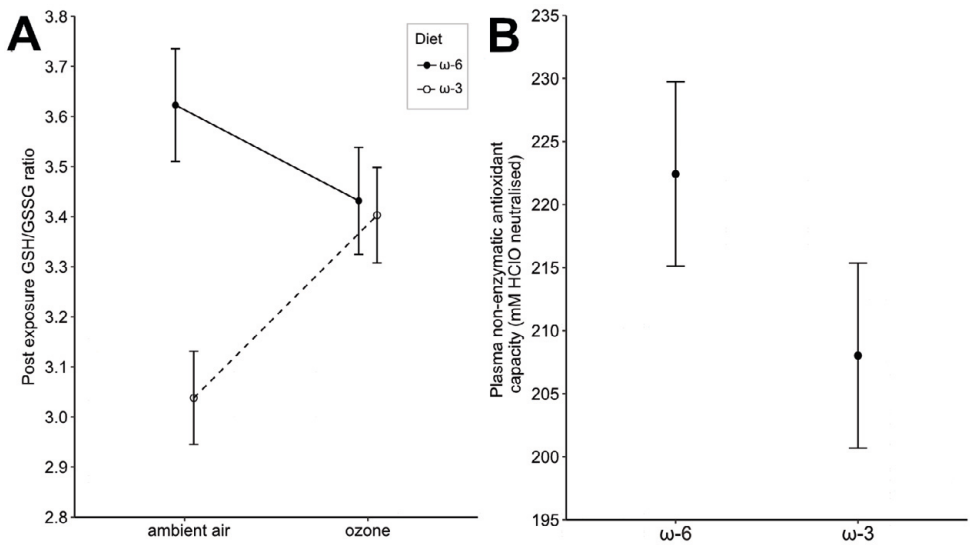

Figure 9. Effects of five days of experimental exposure to ozone/clean ambient air with birds being either on a diet with a high ω6:ω3 polyunsaturated fatty acid (PUFA) ratio (i.e., ω-6, black points) or on a diet with a low ω6:ω3 PUFA ratio (i.e., ω-3, white points) on the the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) (A) and on the levels of non-enzymatic antioxidant capacity (OXY) (B). Means ± standard errors from predicted values of final models are shown. Details in paper IV.

Relative effects of abiotic and biotic territory characteristics on nestling physiology (Paper V)

As we saw in *papers II–IV*, ALAN, anthropogenic noise, air pollutants and diet can have profound effects on nestling and adult physiology. In *paper V,* we went from the laboratory back to the wild with the aim to quantify the relative contributions of different abiotic and biotic territory characteristics on nestling physiology. It is notoriously difficult to manipulate more than one habitat characteristic in a wild setting, but for this study we took advantage of small-scale variation of the different characteristics between territories. Therefore, we performed a correlational study, measuring number of light sources, concentration of $PM_{2.5}$ and NO_2 as air pollutants, ambient temperature, and tree composition, within a great tit territory (radius of 35 m around a nest box), and correlated these abiotic and biotic habitat characteristics to nestling oxidative stress physiology and plasma fatty acid composition. To capture the variation in tree composition we quantified the total number of trees, distinguished between the number of oak, beech, birch and non-native trees and counted the total number of trees species.

First, we performed a principle component analysis (PCA), which revealed that the quantified environmental variables create an urbanisation gradient in the first principle component (PC1). Summarising several components into a single gradient is a widely used approach to quantify effects of urbanisation on wildlife (Blair, 1996; Caizergues et al., 2021; Jimenez-Penuela et al., 2019; McDonnell and Pickett, 1990; Seress et al., 2014; Marzluff, 2017). Even though such an approach has yielded useful information, it is not able to attribute specific variables that compose the gradient to the different organismal effects and hence hinders a thorough mechanistic understanding of the effect dynamics. In line with this argumentation, we found that both the number of oak trees and $PM_{2.5}$ concentration negatively affected antioxidant capacity of great tit nestlings (Fig. 10A and B). These two environmental variables loaded in opposite directions in PC1. Using only a single gradient such as PC1 would likely have obscured the impacts of air pollution and vegetation structure on nestling oxidative stress physiology. The negative impact of oak trees on antioxidant capacity may seem counterintuitive, as oak trees are known to host important nestling invertebrate food sources (Wilkin et al., 2009). One explanation may be that a lower antioxidant capacity is the result of increased ROS scavenging due to increased growth rate, modulated by favourable conditions when more oak trees are present (van Noordwijk et al., 1995; Naef-Daenzer et al., 2000). In support of this explanation, we found a positive correlation between the number of oak trees and nestling body mass and wing length. Alternatively, a negative relationship between antioxidant capacity and number of oak trees (Fig. 10B) may reflect habitat quality, as the number of oak trees in a territory has been linked to caterpillar availability (Jensen et al., 2021). Nestlings from oak-poor territories may have higher antioxidant capacity due to a reduced water intake via caterpillars, due to lack of water in urban areas (Becker And McCluney, 2021), which in turn would

decrease the plasma water content and increase the concentration of antioxidants measured in a blood sample.

Nestling plasma ω6:ω3 PUFA ratio was positively correlated with $PM_{2.5}$ concentration and negatively associated with ambient temperature and number of non-native trees. Due to this biomarker being a ratio and the correlative nature of this study, we cannot draw specific conclusions about the dynamics that changed the ratio. A negative correlation between temperature and ω6:ω3 PUFA ratio may reflect a temperature-dependent structural modification of membranes for higher membrane viscosity at lower temperatures. However, we did not find a correlation between temperature and unsaturation index, and we measured circulating FA instead of tissue FA composition. High $PM_{2.5}$ concentration may indirectly affect the ω6:ω3 PUFA ratio by negatively impacting invertebrate abundance and quality (Blande, 2021) and thus leads to a poorer diet (quality and/or quantity) for nestlings. Similarly, non-native trees have been shown to host fewer invertebrates (Burghardt and Tallamy, 2015; Helden et al., 2012; Jensen et al., 2021) and thus could indirectly affect ω6:ω3 PUFA ratios by providing low quality and quantity food.

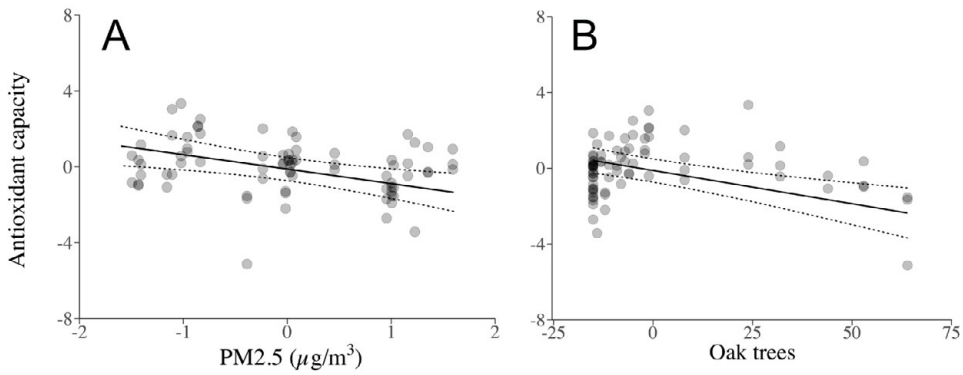

Figure 10. Corrected antioxidant capacity of great tit nestlings depending on environmental variables. Significant correlation between antioxidant capacity with concentration of particulate matter 2.5 ($PM_{2.5}$; A), the number of oak trees within a territory (B). The antioxidant capacity is corrected for uric acid levels and $PM_{2.5}$ and number of oak trees are mean-centred. Dotted lines indicate 95% confidence interval. Details in paper V.

Conclusion and future perspectives

The aims of this thesis were (i) to explore, disentangle, and characterise the single stressor and combined stressor effects of different urban pollutants on avian physiology and behaviour, and (ii) to gain a deeper knowledge of the physiological mechanisms involved in mediating phenotypic responses to the exposure to different anthropogenic pollutants. We performed controlled exposure experiments in the laboratory and the wild and also investigated relative contributions of abiotic and biotic territory characteristics to free-living nestling physiology in a correlative study.

Based on the findings presented in this thesis, we can present some main conclusions:

- ALAN and anthropogenic noise can influence different behaviours separately, but also create an antagonistic, non-additive effect.
- Immune function can be altered by ALAN exposure and the mechanism driving such effects is likely a reduction of night-time melatonin levels.
- Particulate matter, found in an urban environment, affects avian physiology, but soot produced in the lab is probably too "clean" to elicit any effects. On the other hand, lab-produced ozone as an air pollutant seems to be a potent pro-oxidant, that elicits an antioxidant defence response.
- Different dietary ω6:ω3 PUFA ratios, can modulate oxidative stress responses to an oxidising pollutant or act alone, influencing oxidative stress status. Likewise, physiological circulating ω6:ω3 PUFA ratios are influenced by anthropogenic induced environmental change.
- Due to the complex relationship between the different parts of the oxidative stress system and its integration with other physiological processes, such as immune function, responses to stressors are highly context-dependent. The interpretation of findings needs to consider the respective context of the study.
- Investigating and measuring simultaneously several abiotic and biotic factors gives a more profound mechanistic understanding of phenotypic effects, rather than using an urban-rural dichotomy or a single gradient.

Specifically, in paper I, we found that ALAN is a strong driver of behavioural effects, mostly affecting activity related behaviours of zebra finches. Whether the shift of behaviours due to pollutant exposure has any ecological consequences in wild birds needs to be further investigated. It is likely that changes of behaviours due to pollution exposure influence predation risk, foraging duration, body condition and higher-level population and community dynamics. The applicability of our experimental laboratory results to scenarios in the wild may be limited, but studying behavioural multi-stressor responses in the wild may prove difficult.

In paper II, we show mechanistic evidence through which ALAN affects physiology in a wild bird. ALAN can act as a disruptor or suppressor of endocrine processes, which has consequences on downstream physiological processes, such as immune function. Our results suggest that ALAN exposed nestlings either directly lack the resources, or capacity to fight a (simulated) invading pathogen, or that they indirectly adaptively react with a decreased immune response to minimise immune response associated oxidative damage. Ultimately, an altered capacity to mount an immune response due to ALAN exposure may have important implications on future performance and survival.

In paper III, we found unexpected interactive effects of multi-stressor exposure on oxidative stress physiology, giving an impression of a paradoxically more beneficial oxidative stress state under supposedly more stressful situations than under no or single stressor conditions. This indicates that stressful conditions trigger increased release or activity of antioxidants. Whether maintaining increased levels of antioxidants comes at any cost in the long-term has to be determined. Thus, possible trade-offs between competing physiological functions and life-history traits may arise. A suggestive trade-off between the life-history traits immune function and growth was found in paper II. However, in this thesis we did not determine whether the measured responses have fitness consequences. Physiological processes like oxidative stress system and immune function have been previously been linked to survival and fitness (e.g., Bize et al., 2008; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Møller and Saino, 2004). Hence, it is likely that changes that we measured in these physiological traits could explain observed fitness differences between urban and rural populations of birds.

The urban environment is a heterogeneous mixture of different stressors. By comparing only urban versus rural populations and treating the urban environment as one stressor, one is likely to miss the underlying mechanisms that shape phenotypic variation found between and within populations. A holistic approach, which measures and/or manipulates several environmental factors, is necessary to overcome this shortcoming. We did so in paper V by correlating abiotic and biotic territory characteristics to nestling physiology and found effects on for example antioxidant capacity, which would have been obscured by using a “simple” urban versus rural comparison or gradient.

Findings in this thesis highlight that responses of physiology and behaviour can be context-dependent and lead to non-additive effects, but single stressors may for some responses also be the main drivers. Reasons for this diversity of "stressor-to-effect routes" are likely a mixture of uni-, cross- and/or multi-modal perception of, and response to stressors, which has the potential to create complex, sometimes non-additive effects (Halfwerk and Slabbekoorn, 2015; Munoz and Blumstein, 2012). Gaining a better understanding of the dynamics between stressors is crucial to be able to add greater insight into the potential for populations and species to evolve and adapt in the face of environmental change.

There are many more layers of complexity in multi-stressor research for which studies in the urban context can provide a fruitful ground, but discussion of these here would go beyond the scope of this thesis. Here only a few examples of what I think would be highly interesting to explore in the urban context: temporal dynamics of exposure to multiple stressors, mechanisms of non-additive effects on higher temporal and biological scales, the role of transgenerational and maternal effects and species-specific effects.

Finally, measured responses to pollutant exposure in this thesis are likely all plastic responses rather than genetic adaptations to local conditions. First, we used "naive" previously unexposed birds for our laboratory experiments and second, the great tit nestlings used in paper II were all from the same population of rural birds. An exception might be the phenotypic differences measured in paper V, where nestlings sampled in the urban territories might show intrinsic adaptations to a life in urbanised areas (Watson et al., 2017; but see Salmón et al., 2018b). It is thinkable that responses of locally adapted individuals and plastically reacting individuals differ in magnitude and even direction. Therefore, the reason for (not) finding certain effects in studies comparing urban and rural populations, or finding seemingly contradicting results, might be the differential contribution of underlying genetic and/or plastic mechanisms. As an example, hand-raised blackbirds originating from urban and rural sites, differed in a common garden experiment in their oxidative stress response towards chronic stress (Costantini et al., 2014). Urban-originating birds had lower oxidative damage than rural-origin birds, but urban birds also showed lower antioxidant levels, indicating an intrinsic difference in oxidative stress status (Costantini et al., 2014). Common garden and also cross-foster experiments are fruitful future approaches to disentangle whether the phenotypic differences found between urban and rural populations are the result of plastic reactions and/or adaptation (e.g., Costantini, 2014; Davies et al., 2017; Dominoni et al., 2020c; Ouyang et al., 2019; Salmón et al., 2018b).

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Figure 11. Interpretation of a great tit between Skrylle and Malmö (by Philip Downing).

Popular summary

The human population will soon reach eight billion people. Today about 55% of these people already live in cities and this number will grow even higher in the future. Creating new city areas changes the landscape dramatically from natural green habitats to deserts of concrete, where we might find, here and there, little islands of highly managed green space. Traffic and industry exhaust soot and toxic gases into the air, they also produce loud noise, and street, and park lamps illuminate normally dark skies. All these pollutants pose novel challenges to animals living in cities.

Some animals seemingly like cities. They have new food and more abundant food sources, like the left-over French fries from our lunch and the provided bird seeds in the backyards. They also experience warmer temperatures in winters in cities and have therefore a higher chance of survival. Other animals that live in cities are in worse condition than their "friends" in the forest. Researchers studying the differences between animals inside and outside cities also found that some birds sing higher pitched and earlier in the morning. Likely to predominate the loud rush hour sound. Or, city birds have been found to forage late into the night instead of going to sleep, because light from street lamps shines bright enough. Not so visible like behavioural differences between city and rural birds, but still existent, are physiological differences, such as differences in immune function and oxidative stress status. The latter describes a balance act between the body's antioxidant defence system (for example vitamins and carotenoids) and reactive pro-oxidants, which are molecules that like to attack other molecules and by doing so cause damage to DNA, proteins and lipids in membranes (called oxidative damage). The antioxidants job is to clean up the reactive molecules, which are either produced through normal cell metabolism, an immune response or pollutants like ozone and soot. If there are too many of these reactive molecules, and the antioxidants cannot keep up, the damage accumulates and leads to a state called oxidative stress.

We now see all these differences between city and rural animals and can conclude that cities and the pollutants in there have some kind of effect on behaviours and physiology, but most often we do not know exactly which of the many pollutants causes the differences. Is an animal in the city in a bad condition because it breathes polluted air, or because the noise levels are too high? Not fully understanding such questions is problematic, because if we want to, for example, make cities a nicer place to live for us and the wildlife in there, we need to know where to start making

changes. On top of that, we do not yet fully understand the interplay between different pollutants and if the effects are different if an animal is exposed to two or more pollutants than when just exposed to one. So called interactions are a context-dependent effects, and are likely happening in the cases we answer with "it depends". To follow up on the question above; What effect would we find if the animal would additionally live next to a street lamp and be exposed to light at night? Would it be in even worse condition because of the additional light, or could it for example forage more and be better off because it finds more food?

In this thesis, I investigate how and also how much each urban pollutant contributes to behavioural and physiology, like immune function. I also looked if the reaction to an exposure of two or more stressors is more or less than we expect when would add up the two separate effects of the stressors alone. To do so, I used wild great tits that are common both in cities and natural areas and nest in provided boxes, hanging in the city of Malmö, in southern Sweden, and in a nature reserve about 30km outside Malmö. For other more controlled laboratory experiments I used captive zebra finches as representatives of the bird world.

For the first paper, I investigated how exposure to artificial light at night (ALAN) and noise alone and in combination affects bird behaviours like movement, feeding, and resting. I found that ALAN increased resting during the day and noise decreased feeding behaviour, but the combined exposure to noise and ALAN did not change any of these behaviours more than the exposure to only one pollutant. ALAN increased resting compared to no exposure and noise did so too, but to a lesser extent. When the birds were exposed to both ALAN and noise at the same time the effect on resting behaviour was in-between that of ALAN alone and noise alone, indicating that ALAN is a stronger factor influencing resting behaviour than noise.

In the second paper, I wanted to further investigate the effects of exposure to ALAN, this time on the immune system. Melatonin is a hormone, well-known as the "sleep hormone", but it is also an important supporter of the immune system. Animals have high blood levels of melatonin when it is dark, because daylight reduces its production. Therefore, ALAN produced by for example street lamps could pose a problem to animals by reducing night-time melatonin levels, which then could have an effect on the immune system. I installed small LED lamps in great tit nest boxes and found indeed, that after a week of ALAN exposure, the melatonin levels of light exposed nestlings were only half as high as the ones of birds that were living in dark nest boxes. I also measured the immune function of these birds, and from these results I could conclude that ALAN-exposed birds did not respond as strongly as dark-night birds to an immune challenge. This indicates that exposure to ALAN could have negative effects on immune function.

For the third paper, I am going back to a laboratory experiment, where I investigated the effects of this time three different pollutants on oxidative stress status of birds. Birds were exposed to ALAN, traffic noise and/or the air pollutant soot. As for paper

I, we here also asked if the combined exposure to multiple pollutants at the same time has different, perhaps unexpected effects, then the exposure to only one pollutant alone or none. The first surprising result was that air pollution did not cause any effect on the oxidative stress status. Perhaps the soot produced in the laboratory is not as toxic as the actual soot particles found in cities. The second surprising result was that birds exposed to ALAN and noise together had higher levels of antioxidants and lower levels of oxidative damage than expected if the two single effects of the pollutants were added together. These results indicate a stronger response when exposed to multiple pollutants than to only one and highlights that multiple pollutants acting together can give rise to unexpected effects.

In addition to air, noise and light pollution, animals living in cities often eat food that is different to their natural food sources. For example, human produced food and also seeds and nuts provided at bird feeders, contain a different composition of nutrients that can cause and enhance the inflammation in the body. In paper IV, I was interested in whether eating a diet containing high ratios of inflammation causing components (i.e., high omega (ω)6 to ω3 polyunsaturated fatty acid (PUFA) ratio; high-ratio diet) creates a disadvantage over eating a diet with a low ratio of inflammation causing components (i.e., low ω6: ω3 ratio; low-ratio diet); first by already being on this diet and second when a bird is additionally exposed to air pollution, which can also create inflammation in the body. In this experiment, I used ozone as the target air pollutant. I was surprised to find that a low-ratio diet decreased the capacity to fight reactive molecules that would create oxidative damage, compared to a high-ratio diet. This effect was independent on ozone levels. Similarly, birds on a low-ratio diet showed higher current oxidative stress than birds on a high-ratio diet, but this effect was only visible in conditions without ozone. When birds were exposed to ozone, which I expected to increase oxidative stress particularly for birds on a high-ratio diet, I found no difference between the two diet groups. Surprisingly, there was no difference in current oxidative stress levels between the two diet groups, regardless birds on a high-ratio diet indicating lower levels of oxidative stress in these birds. The findings show that there are more complex relationships between diet and air pollution than expected and further studies should investigate the role of different diet compositions when it comes to changing responses to pollutants.

Finally, in the fifth paper, I went back to asking my original question of which pollutant is causing which effect, but this time in the wild. To answer that question, we measured different urban characteristics, like number of light sources, number and type of trees, concentration of air pollution and temperature in a territory around nest boxes of great tits and looked at how they affect nestling physiology. First, we could show that measuring different variables gives us more detailed answers than if we would have compared only city and rural birds. Second, we learned that air pollution negatively affects nestling antioxidant defence and that nestling body mass is dependent on the number of light sources and the type of trees (native or non-

native) in the territory. If there were many light sources and non-native trees around the nest box nestlings were heavier, but with few light sources and less non-native trees around, nestlings were of smaller body mass. This is perhaps caused by a complex shifting effect ALAN on trees sprouting leaves in spring, which then provide caterpillars as food for the nestlings.

In summary, the results found in my thesis show that exposure to city pollutants poses challenges to birds´ behaviour and physiology. Single and combined exposure to different pollutants can lead to different effects, which in some cases was unexpected - if we compared to results from on pollutant or made a simple urban-rural comparison. This highlights the need for further studies which include multiple stressors and complex interactions.

Populärvetenskaplig sammanfattning

Globalt når vi snart en befolkningsmängd på åtta miljarder människor. Idag bor cirka 55 % av dessa människor i städer, som i framtiden kommer bli både fler och större. Att skapa nya stadsområden förändrar landskapet dramatiskt, från naturliga gröna livsmiljöer till öknar av betong där endast små oaser med välskötta grönområden återstår. Trafik och industri släpper ut sot och giftiga gaser i luften, vilket skapar både luftföroreningar och höga bullernivåer i städerna. Dessutom är den normalt mörka himlen i staden upplyst av olika gat- och parklampor. Alla dessa föroreningar utgör nya utmaningar för de djur som lever i städer.

Vissa djur verkar trivas i staden. De har rikliga källor av olika sorters mat, från pommes frites som tappas på gatan till olika sorters fröer och nötter på fågelborden. De upplever även ett mildare klimat under vintrarna, vilket gör att djur i städerna kan ha en större chans att överleva. Men trots detta är det många stadslevande djur som är i sämre skick än sina "vänner" i skogen. Det finns också arter som inte alls trivs, eller kan leva, i städerna. Faktum är att för många djur är staden inte en lämplig livsmiljö. I takt med en ökad global urbanisering har forskning kring hur stadsmiljön påverkar olika arter blivit allt mer intensiv, och man har redan noterat flera viktiga effekter. Till exempel sjunger vissa stadslevande fågelarter tidigare på morgonen och med en ljusare ton, sannolikt för att kunna överrösta stadens buller. Andra arter drar nytta av gatubelysningen och har setts äta och söka föda till sent in på natten istället för att sova.

Fysiologiska skillnader mellan stads- och landsbygdsfåglar är inte lika synliga som de ovan nämnda skillnaderna i beteenden, men nog så viktiga. I synnerhet den fysiologi som är kopplad till kroppens försvar mot föroreningar, som immun- och antioxidantförsvaret. Det senare kopplas till oxidativ stress, vilket är en balansakt mellan skyddande antioxidanter (till exempel vitaminer) och reaktiva, så kallade pro-oxidanter, som är molekyler som gärna ”attackerar” andra molekyler och på så sätt orsakar skador på arvsmassan (DNA). Antioxidanternas uppgift är att rensa upp de reaktiva molekylerna, som antingen produceras direkt av kroppen själv (via vår ämnesomsättning eller immunförsvar) eller kommer från luftföroreningar, som ozon och sot. Om antioxidanterna inte hinner städa upp de reaktiva molekylerna ackumuleras skador, vilket är det tillstånd som kallas oxidativ stress.

Även fast effekter på beteende och fysiologin har kunnat påvisas vid direkt jämförelse mellan djur som lever i staden eller på landsbygden kvarstår frågan om

exakt vilken eller vilka miljöfaktorer som orsakar skillnaderna. Är ett djur i staden i dåligt skick för att det andas in förorenad luft eller för att ljudnivåerna är för höga? Att förstå orsakerna är viktigt för att kunna skapa en bättre och mer hållbar stadsmiljö för djur som lever där, men även för oss själva. Utöver det förstår vi ännu sämre hur olika föroreningar samverkar, och om effekterna en eller flera.

I den här avhandlingen har jag undersökt hur olika föroreningar associerade med stadsmiljö påverkar beteende och fysiologi hos fåglar. Jag undersökte även om effekten av två eller flera föroreningar var högre eller lägre än vad man skulle förvänta sig av effekten av en enskild förorening. För att studera detta använde jag mig av både vilda talgoxar och buruppfödda zebrafinkar. Talgoxen är vanligt förekommande och häckar gärna i holkar, både i städer och naturområden. För mer kontrollerade laboratorieexperiment använde jag zebrafinkar som representanter för fågelvärlden.

I den första studien (artikel I) undersökte jag hur exponering av artificiellt ljus på natten (ALAN) och buller, antingen enskilt eller i kombination, påverkar beteenden som aktivitet, födointag och vila, hos zebrafinkar. ALAN ökade zebrafinkarnas vila på dagen och buller minskade födointaget, men den kombinerade exponeringen förändrade inte något av dessa beteenden mer än exponeringen för endast en av föroreningarna. Till exempel påverkade både ALAN och buller enskilt vila, men effekten var mycket mindre för buller. När fåglarna exponerades för både ALAN och buller samtidigt påverkades vilobeteendet mindre än vid exponering för endast ALAN, men mer än vid exponering av endast buller. Detta betyder att ALAN har en starkare effekt på vilobeteende än vad buller har.

I den andra studien (artikel II) undersökte jag effekterna av exponering för ALAN, denna gång på talgoxars immunförsvar. Melatonin, som ofta kallas för "sömnhormonet", produceras framförallt under dygnets mörka timmar. Hormonet har också en viktig funktion i immunförsvaret, och därför kan ALAN från till exempel gatlyktor försämra både sömnen och kroppens försvar mot sjukdomar. Jag installerade små LED-lampor i holkar med häckande talgoxar och inom en veckas exponering sjönk melatoninnivåerna hos de ljusexponerade fågelungarna till hälften i jämförelse med de ungar som inte var exponerade för ljus nattetid. Jag mätte även olika markörer av deras immunförsvar, vilka visade att fågelungar exponerade för ALAN inte hade lika stark immunrespons. Detta tyder på att exponering för ALAN har negativa effekter på immunförsvaret.

I den tredje studien i avhandlingen (artikel III), utförde jag återigen ett laboratorieexperiment med zebrafinkar. Denna gång undersöker jag effekterna av tre olika föroreningar på oxidativ stress, antingen enskilt eller i kombination. Fåglar utsattes för ALAN, trafikbuller och/eller luftföroreningen sot. Precis som i artikel I undersökte jag här om den kombinerade exponeringen för flera föroreningar samtidigt har andra, kanske oväntade effekter, än exponeringen för endast en förorening. Det första överraskande resultatet var att sot inte orsakade någon

oxidativ stress (för de markörer vi använde). En förklaring kan vara att det sot som produceras på laboratoriet inte är lika giftigt som de faktiska sotpartiklarna som finns våra städer. Det andra överraskande resultatet var att fåglar som exponerades för ALAN och buller samtidigt hade högre nivåer av antioxidanter och lägre nivåer av oxidativ stress, jämfört med om de enskilda effekterna av föroreningarna summerades. Dessa resultat indikerar en starkare respons när de utsätts för flera föroreningar än för endast en och visar att flera föroreningar som verkar tillsammans kan ge upphov till oväntade effekter.

Förutom att djur i städerna påverkas av luft-, buller- och ljusföroreningar har de ofta tillgång till mer mat, som också skiljer sig från den de hittar i naturen. Denna mat innehåller ofta färre näringsämnen och/eller en annan sammansättning, som till exempel olika typer av fleromättade fettsyror (som tillexempel förhållandet mellan omega-6 och omega-3) vilket kan orsaka inflammation i kroppen. I artikel IV undersökte jag om en diet innehållande en hög proportion av inflammationsorsakande komponenter (d.v.s. mer omega-6 till omega-3 fettsyror; högkvots-diet) är sämre för fåglar än en diet med låg andel inflammationsorsakande komponenter (d.v.s. mer omega-3 än omega-6; lågkvots-diet). Vidare undersökte jag den simultana effekten av diet och exponering av luftföroreningar. I det här experimentet använde jag ozon som en luftförorening. Något förvånande fann jag att en lågkvots-diet minskade förmågan att bekämpa reaktiva molekyler jämfört med en diet med hög kvot, och effekten var oberoende av ozon. På samma sätt visade fåglar på en diet med låg kvot högre oxidativ stress, men denna effekt var endast synlig under förhållanden utan ozon. När fåglarna exponerades för ozon, vilket förväntades öka oxidativ stress, fann vi ingen skillnad mellan de två dietgrupperna, vilket tyder på lägre nivåer av oxidativ stress hos dessa fåglar. Resultaten visar att det finns mer komplexa samband mellan kost och luftföroreningar än förväntat och ytterligare studier bör undersöka vilken roll olika kostsammansättningar spelar för att försvara sig emot föroreningar.

Slutligen, i den femte studien (artikel V), gick jag tillbaka till att ställa min ursprungliga fråga om vilken förorening som orsakar vilken effekt, men den här gången i det vilda, men begränsat till staden. Vi mätte olika urbana faktorer runt flera holkar med häckande talgoxar och testade hur dessa påverkade immunförsvaret och det oxidativa försvaret hos ungarna. De urbana faktorer som inkluderades var antal ljuskällor, koncentrationen av luftföroreningar, antal och typ av träd, och temperatur. Det första målet med studien var att undersöka om en mer detaljerad miljöbeskrivning i form av våra olika faktorer ger en bättre förståelse för olika fysiologiska reaktioner snarare än den mer traditionellt använda jämförelsen mellan stads- och landsbygdsfåglar. Vi kunde visa att luftföroreningar påverkade antioxidantförsvaret negativt och att talgoxarnas vikt var beroende av mängden ljuskällor och typen av träd (inhemska eller icke-inhemska) i fåglarnas omgivning. Om det fanns många ljuskällor och icke inhemska träd runt holken var ungarna tyngre (vilket är en indikation på bättre hälsa), men med få ljuskällor och mindre

främmande träd runt omkring, var ungarna lättare. Orsaken till denna effekt är inte klarlagd, men tidigare studier har visat att ALAN kan påverka lövsprickning på våren vilket i sin tur kan påverka mängden fjärilslarver vilket är talgoxungars huvudföda på våren.

Sammanfattningsvis, resultaten i min avhandling visar att exponering för föroreningar i staden påverkar fåglarnas beteende och fysiologi. Olika föroreningar verkar leda till olika effekter, effekter som ibland inte hade förväntats om man bara undersökt en förorening eller gjort en enkel jämförelse mellan stad och landsbygd. Detta understryker behovet av ytterligare studier som inkluderar flera miljöföroreningar och dess komplexa interaktioner.

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