URBAN EVOLUTION IN THE WATER FLEA *DAPHNIA MAGNA*

Kristien I. BRANS

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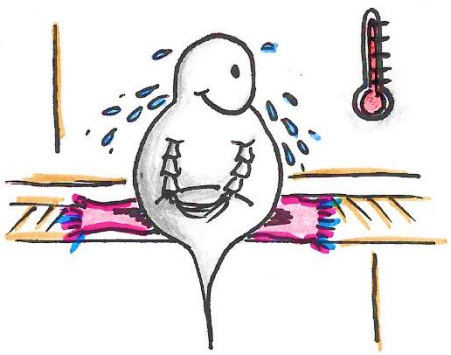
KU Leuven CT_{MAX} test



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General Introduction

Cities: the main habitat where human populations thrive and an inseparable part of modern day landscapes. Frank Sinatra's 'New York' embodied the hopes and dreams that come with never-sleeping cities; growth, wealth, success, and fast, enriched, lives. Forty years later, Alicia Keys and Jay-Z sang about the same "concrete jungle where dreams are made up", and apparently, "big lights will inspire us". That 'big city life' and, amongst others, its lights did inspire us, environmental ecologists and evolutionary biologists across the world. Urban development and human activities come at a clear environmental and biological cost. Currently, urban environments cover ca. 3% of the Earth's land surface and 50% of human population lives in cities, a percentage that is expected to increase to 66% by 2050 due to increased human population growth and urban development (UN, 2014). Urbanization has led to substantial abiotic and biotic changes in the natural and urban environment (Vitousek *et al.*, 1997; Grimm *et al.*, 2008a,b; Alberti, 2015; Parris, 2016; Alberti *et al.*, 2017a,b), changing Earth's ecosystem dynamics profoundly. Cities and suburban areas are distinct hotspots of disturbances (Kaye *et al.*, 2006; Grimm *et al.*, 2008a,b; Parris, 2016) such as warming (known as 'the urban heat island' effect; Oke, 1973; Ward *et al.*, 2016), eutrophication (Yu *et al.*, 2012; Waajen *et al.*, 2014; Toor *et al.*, 2017), chemical contamination (Paul & Meyer, 2001; Gorissen *et al.*, 2005; Gilliom, 2007; Roux & Marra, 2007), habitat fragmentation (Forman & Alexander, 1998; Marzluff, 2001; Moilanen & Nieminen, 2002; Cane *et al.*, 2006; Grimm *et al.*, 2008a; Elmqvist *et al.*, 2016; Parris, 2016), light and noise pollution (Halfwerk & Slabbekoorn, 2015; McMahon *et al.*, 2017), hydrological imbalance, and a high introduction rate of exotic and invasive species (Riley *et al.*, 2005; Shochat *et al.*, 2010; Gaertner *et al.*, 2017).

Intrigued by all these urbanization-driven abiotic and biotic changes in the landscape, ecologists put on their urban boots and followed Frank and Alicia into the urban maze to study biological life in cities, mainly focusing on species abundances and biodiversity (Beissinger & Osborne, 1982; McDonell & Pickett, 1993; Blair, 1996; Blair & Launer, 1997; Gering & Blair, 1999; Alberti & Marzluff, 2004; McKiney, 2006; Parris, 2006; Shochat *et al.*, 2006; Shochat *et al.*, 2010; Ferraguti *et al.*, 2016; Piano *et al.*, 2017; Leong *et al.*, 2016, 2017; Merckx *et al.*, 2018; Seress *et al.*, 2018). An often reported phenomenon in urban ecology, for example, is the homogenization of plant and animal communities as a consequence of decreased species diversity due to habitat loss, degradation and homogenization, in combination with a higher incidence of introductions of non-native and exotic species (McIntyre, 2000; Blair, 2001; McKinney 2006, 2008; Devictor *et al.*, 2007; McIntyre, 2014; Deguines *et al.*, 2016; Morelli *et al.*, 2016; Schmidt *et al.*, 2017 for microbial communities). Nevertheless, there are 'roses' and even 'sunshine' in the city. If natural habitats are not completely replaced by urban or industrial areas, a number of organism groups will face urbanization by, for example, moving out and dispersing to suitable semi-natural or natural habitats (i.e. 'urban avoiders'). On the other hand, some might respond to these alternative urban selection pressures by showing resilience and adaptive changes ('urban adapters'/'urban utilizers'), which can even lead to some groups benefiting from urban

environments (e.g. cliff- and cavity-nesting birds like the rock pigeon, house sparrows, and house finces), or even exploiting the urban scenery (‘urban exploiters’/’urban dwellers’) (McKinney, 2002; Kark *et al.*, 2007; Goertzen & Suhling, 2013; Pinel-Allloul & Mimouni, 2013; Fischer *et al.*, 2015). Given that they have broader environmental tolerance limits and could benefit from local extinctions or disappearance of specialist species in cities, the latter are often generalist species (Owens & Bennet, 2000; Marvier *et al.*, 2004; Julliard *et al.*, 2006).

Evolution in nature can occur fast and at much shorter time scales than previously thought. Consequently, evolutionary biologists found a new playground in the city, leading to a currently blooming field in evolutionary biology, i.e. ‘urban evolution’ (Donihue & Lambert, 2015; Johnson & Munshi-South, 2017). Urbanization-associated stressors are selection factors, impacting the relative fitness of individual phenotypes via organismal traits such as life history, physiology, behavior, and immunology, which can drive adaptive evolution (Palumbi, 2001; Chown & Duffy, 2015; Donihue & Lambert, 2015). Over the past five to ten years urban evolutionary studies emerged at a high rate, revealing such evidence of an ‘urban signature’ in both neutral and adaptive evolutionary trajectories of plants (Cheptou *et al.*, 2008; Yakub & Tiffin, 2016; Johnson *et al.*, 2015; Thompson *et al.*, 2016), vertebrates (e.g. birds, rodents, amphibians, reptiles; Partecke *et al.*, 2006; Costantini *et al.*, 2014; Winchell *et al.*, 2016; Harris & Munshi-South, 2017), invertebrates (damselflies and dragonflies, spiders, crustaceans, ants; Diamond *et al.*, 2017b, 2018a,b; Tüzün *et al.* 2015; Brans *et al.*, 2017a,b), and micro-organisms (McLean *et al.*, 2015).

While they are crucial contributors to regional biodiversity, ecosystem functioning, and human well-being, aquatic ecosystems such as ponds and shallow lakes, have received considerably less attention in general (Céréghino *et al.*, 2008; Hill et al 2018a), and more particular in urban ecological and evolutionary studies (Hassall, 2014; Thornhill *et al.*, 2018). During my PhD, I set out to study the impact of urbanization on evolutionary processes and its eco-evolutionary consequences with a clear focus on aquatic ecosystems. In this general introduction of my dissertation, I will provide a short overview of the most important landcover- and microclimate- associated abiotic and biotic consequences of urbanization. I will introduce the fields of ‘urban ecology’ (Parris, 2016), ‘urban evolution’ (Donihue & Lambert, 2015; Johnson & Munshi-South, 2017) and ‘eco-evolutionary dynamics’ (Alberti, 2015; Hendry, 2017). Finally, I will elaborate on the current research gaps, situate the background of the study system and area, and provide an overview of the outline and aims of my dissertation.

1 Urbanization

The current day era is officially defined as the 'Anthropocene' (Crutzen & Steffen, 2003; Steffen *et al.*, 2011), due to the profound impacts of human activity and human population growth on the natural environment since the industrial revolution (ca. 1760) (i.e. including industrialization, intensive agriculture and fishery activities, animal husbandry, urbanization, and the development of nuclear technologies). Over half of the human population resides in urban areas, which is predicted to rise to 66% by 2050 (Seto *et al.*, 2010, 2012; UN, 2014). Due to the specific characteristics of urban development and human activities, the urban 'anthrobiome' is associated with strong abiotic and biotic environmental alterations, generating a complex, yet strikingly 'similar' terrestrial environment across cities worldwide (McKinney, 2006; Pouyat *et al.*, 2007; Pickett *et al.*, 2011; Groffman *et al.*, 2014). Urbanization is commonly associated with warming, eutrophication, pollution, habitat fragmentation, and the introduction of non-native and exotic species (Kaye *et al.*, 2006; Pouyat *et al.*, 2007; Grim *et al.*, 2008a,b; Mcdonald *et al.*, 2008; Gaston *et al.*, 2015; Parris, 2016). Road networks across the landscape furthermore contribute to the extension of human-associated disturbances into more remote places (Trombulak & Frissell, 2000). I will detail these urbanization-driven environmental changes in the next paragraphs and elaborate more on the possible impacts on organisms.

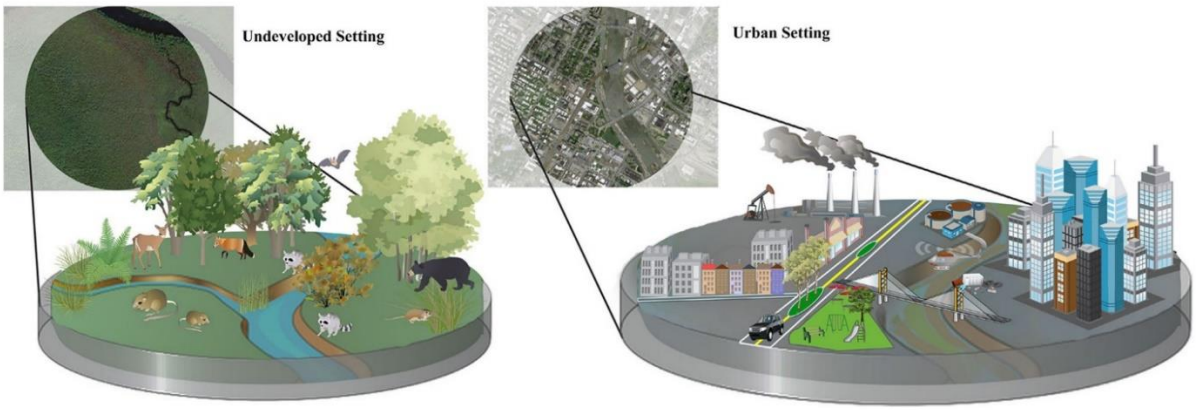

Figure 1. General representation of typical rural (here: undeveloped) and urban areas. From Donihue & Lambert (2015).

1.1 The urban environment

While the term 'urbanization' is conceptually straightforward, urbanization dynamics, both spatially and historically, differ strongly across larger geographical regions (e.g. Europe versus Asia or the United States). Metrics to define urbanization in scientific literature are rather broad (e.g. based on population densities, urban development rate, population development, built-up area, distance to the urban core, number of households per square kilometer, etc.; Chan & Hu, 2003; Kesteloot, 2003; Birch & Wachter, 2011; Tempels *et al.*, 2012; UN, 2014; Schafazideh-Moghadam & Helbich, 2015; Johnson

& Munshi-South, 2017;). We here term urbanization as the combined occurrence of physical, chemical, and biological 'urban-associated conditions' (e.g. increased built-up area, habitat fragmentation, warming, eutrophication, chemical pollution, and introduction of exotic species). In this study, we use built-up area in the surroundings of the sampling point as a proxy for the intensity of urban conditions (see further 3.1, 3.2, 3.4). We define 'cities' as urban centers, or cores, in which urban conditions are on average more present or extreme. Yet, 'natural conditions' are not necessarily absent from cities, and, similarly, 'urban conditions' are not necessarily absent from rural areas.

1.1.1 Climate: cities are 'heat islands'

The most known and globally recurring stressor associated with urbanization is the 'urban heat island effect' (UHI; Oke, 1973; Arnfield, 2003; Imhoff *et al.*, 2010). Cities build up heat compared to natural areas due to the high thermal conductivity of buildings, the use of construction materials with low albedo values (and thus high warmth absorption capacity). Additionally, vegetation is often removed, which reduces evapotranspiration and consequently environmental cooling and temperature buffering (Taha, 1997; Arnfield, 2003; Rosenzweig *et al.*, 2009; Soltani & Sharifi, 2017). Human transportation and the frequent use of air-conditioning installations generate additional heat (Fan and Sailor, 2005; Rosenzweig *et al.*, 2009). The phenomenon of urban warming elicits a profound microclimatic shift in the ambient and surface temperatures along rural-to-urban gradients, which have now been monitored in detail via satellite and remote sensing data or even fine-scale temperature measurements (Zhou *et al.*, 2004; Imhoff *et al.*, 2010; Zhao *et al.*, 2014; Kaiser *et al.*, 2016; Li *et al.*, 2018). Urban heat islands can drive a range of temperature differences depending on the city size and population density. Usually UHIs range between 1 °C and 4 °C (Sultani & Sharifi, 2017; e.g. for Flanders, the study area of this thesis, see De Ridder *et al.*, 2015), but extremes of +7-10 °C have been recorded (Oke, 1973; Gartland, 2008; Kolokotsa *et al.*, 2009). Urban heat islands can occur at regional, but even local, and sub-local scale (e.g. Kaiser *et al.*, 2016).

Consequences of urban warming for biological communities can arise from different aspects of the UHI phenomenon. This not only includes higher average temperatures, but also the extension of growing seasons given that urban areas often reach a thermal climate suitable for growth earlier compared to rural areas. Extended growing seasons in cities have been commonly reported in UHI studies (Zhang *et al.*, 2004; San Martin Y Gomez & Van Dyck, 2012; Schatz & Kucharik, 2016; Zipper *et al.*, 2016) and are associated with strong shifts in plant and animal phenology (see 1.2). The earlier onset of vegetation growth in urban areas has even been applied as a tool to monitor urban heat island effects (Yuan & Bauer, 2006; Jochner *et al.*, 2013; Jochner & Menzel, 2015). In addition to increases in average temperatures and longer growing seasons, urban cores are also expected to exacerbate the occurrence and intensity of extreme temperature events. For example, heatwaves, but also temperature fluctuation and variation patterns (Li & Bou-Zeid, 2013; Ward *et al.*, 2016; Wouters *et al.*, 2017; Zhao

et al., 2018), occurring as a consequence of climate change (Meehle & Tebaldi, 2004; Wouters *et al.*, 2017), will be intensified in cities, which can substantially increase physiological stress on a wide range of organisms (Jentsch & Beierkuhnlein, 2008; Sentis *et al.*, 2013a, 2017; Stoks *et al.*, 2017).

While the majority of freshwater lakes is considered as a tool to buffer and mitigate the urban heat island effect (i.e. 'urban blue space', together with the use of vegetated areas, i.e. 'urban green space'; Tzoulas *et al.*, 2007; Völker *et al.*, 2013; Gunawardena & Kershaw, 2016, 2017; but see recently Sun *et al.*, 2018), anthropogenic warming has been shown to impacts streams, affecting average temperatures, but also daily temperature variation (Paul & Meyer, 2001; Herb *et al.*, 2008). There is a strong correlation between air and water temperature (Rollinson & Rowe, 2018), and a higher likelihood of warm run-off to enter the freshwater systems in urban (e.g. during summer storms; Herb *et al.*, 2008; Hester & Bauman, 2013; Somers et a., 2013; but see Deletic, 1998) compared to rural areas. Therefore, urban lakes and stream ecosystems are expected to suffer from a similar urbanization-driven warming as terrestrial ecosystems, including increased average temperatures and temperature variability due to heat-release from the surrounding built-up area. Yet, empirical evidence is limited, especially for small freshwater ecosystems such as ponds and pools (see further). Due to their smaller size, simple morphology, the use of anthropogenic substrates such as concrete or black plastic, and the surrounding urban matrix (e.g. reduced shading by trees), solar irradiation can potentially penetrate deep into the water column. This can additionally increase temperatures throughout the pond during warm summer days, limiting vertical thermal stratification (i.e. cooler temperatures at the bottom of the ponds versus warmer temperatures at the water surface). Organisms inhabiting those smaller freshwater ponds and pools in cities might thus be substantially affected by warming.

Upon warming, oxygen-limitation for aquatic organisms additionally increases drastically in water due to the lower solubility of oxygen (Bozinovic & Pörtner, 2015; Horne *et al.*, 2015; Chown & Duffy, 2015; Pörtner *et al.*, 2017; Hoefnagel *et al.*, 2018). Additionally, temperature-driven increases in microbial respiration and the mineralization of organic matter positively feed back to oxygen consumption and thus hypoxia (Müller *et al.*, 2012; Meire *et al.*, 2013; Jenny *et al.*, 2016). Therefore, temperature-driven hypoxia-stress due to urbanization and climate change is hypothesized to substantially impact freshwater biota (Daufresne *et al.*, 2009; Saari *et al.*, 2018).

1.1.2 Habitat fragmentation and destruction

Urbanization is intrinsically associated with a conversion from natural and agricultural to urban land cover, resulting in fragmented natural habitats (Marzluff & Ewing, 2001; Luck & Wu, 2002; Angel, *et al.*, 2012; Elmqvist *et al.*, 2016; Parris, 2016). For many species this involves a complete destruction of the habitat and consequently often a direct loss of biodiversity (Fahrig, 2003). Although habitat fragmentation and destruction in terrestrial ecoregions strongly impacts biodiversity upon urbanization, urban areas only cover 3% of the global land surface area (i.e. 'global urban land' as in Liu *et al.*, 2014).

While this percentage is low, most ecoregions impacted by urbanization are concentrated along coastal areas or on islands. These are generally characterized by high levels of endemism and thus valuable for biodiversity (Ricketts & Imhoff, 2003; Ricketts *et al.*, 2005; Mcdonald *et al.*, 2008; Seto *et al.*, 2012; Güneralp *et al.*, 2013). Historical urbanization around the Mediterranean Basins and Atlantic Forest Area increased susceptibility of these biodiversity hotspots to species losses and endangerments. For example, currently 60% of South-America's urban landscapes are located in highly biodiverse regions (Laurance *et al.*, 2002; Mcdonald *et al.*, 2008). Additionally future prospects on urbanization indicate strong increases in urbanization in almost all ecoregions, including the tropics, which are considered one of the most biodiverse ecosystems in the world (Laurance *et al.*, 2002; Mcdonald *et al.*, 2008).

Next to direct species losses, habitat fragmentation and roads disturb species directly and indirectly via altered population dynamics. These result from smaller habitat size (Lande 1988; Andrén & Delin, 1994; Moilanen & Nieminen, 2002), reduced dispersal events and gene flow as a result of both increased isolation of habitats and higher amounts of unsuitable urban matrix between patches (Watts *et al.*, 2004; Cheptou *et al.*, 2008; Sato *et al.*, 2014), and increased edge effects in remnant patches. The construction of roads, highways, and other transportation systems increases additionally the penetration radius of urbanization- and human-associated activities and their detrimental consequences for nature to even the most remote places on Earth (Forman, 2000; Trombulak & Frissel, 2000; Wheeler *et al.*, 2005; Coffin 2007; Mcdonald *et al.*, 2008). Habitat removal and fragmentation not only impacts terrestrial habitats and species. Aquatic ecosystems suffer from damming, removal of freshwater corridors such as small streams, ditches, and creeks. Additionally, the low suitability of urban built-up matrix impedes dispersal of semi-aquatic and aquatic organisms (Dudgeon *et al.*, 2006; Fuller *et al.*, 2015). These processes increase geographic and physical isolation of terrestrial and freshwater ecosystems, reducing effective connectivity between patches (Gledhill *et al.*, 2008; Prugh *et al.*, 2008; Dolgener *et al.*, 2012), and increasing the probability of genetic drift and neutral evolution (see 1.2.2).

1.1.3 Eutrophication

Eutrophication in urban settlements affects both soil and water nutrient concentrations (nitrogen, carbon, and phosphorous) and is strongly associated with atmospheric depositions originating from vehicle emissions of CO_2 and NO_x (Grimm *et al.*, 2008a,b; Rao *et al.*, 2014; Decina *et al.*, 2017), increased usage of phosphorous-containing household products entering via sewer systems (Suwarno *et al.*, 2014; Kundu *et al.*, 2015), surface run-off contaminated with fertilizers (Yu *et al.*, 2012; Decina *et al.*, 2017), and lastly nutrient leakage from septic tanks (Moore *et al.* , 2003; Withers *et al.*, 2011, 2014). While urbanization-driven eutrophication tends to increase during spring, indicating a strong contribution of human-associated use of fertilizers during this period (Decina *et al.*, 2017), eutrophication levels often strongly depend on the system (e.g. ponds and streams with sewer inlet, ponds and streams in parks that are heavily managed, isolated streams and ponds in close proximity of

traffic). Eutrophication is known to strongly impact freshwater systems directly (Scheffer *et al.*, 1993; Smith *et al.*, 1998, 2006; Cook *et al.*, 2018). Increased nutrient concentrations can boost algae growth during spring and summer and alter trophic dynamics (Scheffer *et al.*, 1993; Jeppesen *et al.*, 2000, 2005; Moss *et al.*, 2011; Straile, 2015). The deterioration of these valuable ecosystems in urban areas due to algae blooms is mostly associated with increases in eutrophication (Waajen *et al.*, 2014). A potential health hazard of eutrophication mainly lies in the occurrence of cyanobacterial blooms, which dominate under high temperature and high nutrient conditions (Kosten *et al.*, 2012; Paerl & Paul, 2012; Rigosi *et al.*, 2014). Indirectly, eutrophication, via its stimulation of algae growth, and thus increased oxygen consumption, can feed back to hypoxia (Jenny *et al.*, 2016).

1.1.4 Pollution

Urbanization is associated with numerous forms of pollution affecting the terrestrial and aquatic environment (Davis *et al.*, 2001; Crutzen, 2004; Kaye *et al.*, 2006; Parris, 2016). Among the most important are chemical (metals, pesticides, endocrine disrupters; Paul and Meyer, 2001; Gorissen *et al.*, 2005; Walsh *et al.*, 2005; Roux & Marra, 2007; Metcalfe *et al.*, 2010; Hayzoun *et al.*, 2014; Lambert & Skelly, 2016) and inorganic compounds (e.g. salts; van Meter *et al.*, 2011, 2014; Dugan *et al.*, 2017; Kaushal *et al.*, 2018). Especially freshwater ecosystems in urban areas are considered to suffer from these forms of pollution, via the increased inflow of pollutants via surface run-off (Paul & Meyer, 2001; Beasley & Kneale, 2002; Wenger *et al.*, 2009; Klečka et al, 2010; Qin *et al.*, 2010; Hassall, 2014; Morrissey *et al.*, 2015; Wang *et al.*, 2016; Wang *et al.*, 2018).

Plastics and anthropogenic litter are another form of urbanization-associated pollution, generating clear direct health hazards for wildlife (entanglement, ingestion), as well as indirectly via their breakdown under influence of physicochemical processes (mainly UV-radiation, temperature, O2; Andrady, 2011; Gewert *et al.*, 2015; Klein *et al.*, 2018) and bio-degradation (Klein *et al.*, 2018). This degradation of larger plastics into small particles is expected to be particularly problematic for the aquatic environment, where micro- and nanoplastics build up and affect biological life as nanoparticles can bio-accumulate higher up the food chain (Setälä *et al.*, 2014; Dris *et al.*, 2015; Baldwin *et al.*, 2016; Campbell, 2017; Kramm & Volker, 2018). This bio-accumulation has been empirically shown in marine ecosystems (Derraik, 2002; Cole *et al.*, 2011; Hammer *et al.*, 2012).

Lastly, while different in form, noise (Doygun & Gurun, 2008; Slabbekoorn & Ripmeester, 2008; Manea *et al.*, 2017) and light pollution (Longcore & Rich, 2004; Lyytimäki, 2013; Mazor *et al.*, 2013; Gaston *et al.*, 2015 and references therein; Tamir *et al.*, 2017) are directly linked to cities due to human presence (vehicles, motors, construction sites) and their activity patterns (night life, traffic lights, etc.). Noise and light pollution (including polarized light, see Horváth *et al.*, 2009) mainly intervene with life history and phenology of animals and plants (Kempenaers *et al.*, 2010), behavioral traits (Luarte *et al.*, 2016; Silva *et al.*, 2017; Ouyang *et al.*, 2015, 2017), and sensory and signaling processes

(Miller, 2006; Francis & Ortega, 2009; Horváth *et a;l.*, 2009; Hanna *et al.*, 2011; Nemeth *et al.*, 2013; McMahon *et al.*, 2017). The consequences of noise and light pollution can vary across levels of biological organization (species, population, individual level) but are detected across aquatic and terrestrial ecosystems. In response to noise and light, researchers observed substantial species declines (Hölker *et al.*, 2010; Kyba & Hölker, 2013). At the individual and population level, altered fecundity and stress physiology (Isaksson, 2015; Raap *et al.*, 2017, 2018), disturbed mating, shifts in circadian rythms (Horváth *et al.*, 2009; Raap *et al.*, 2016, 2017; Sun *et al.*, 2017), and altered migration patterns (Longcore & Rich, 2004; Navara & Nelson, 2007; Mazor *et al.*, 2013) were found. Exposure to noise has recently even been reported to affect predation efficiency in damselfly larvae (Villalobos-Jiménez *et al.*, 2017), although empirical evidence on urban populations being affected similarly is so far still absent. In urban areas, light-polarizing surfaces are considered an 'ecological' trap for polarotactic and polarization-sensitive animals, such as mayflies, dragonflies and damselflies (see Horváth *et al.*, 2009 and references therein). Reflective surfaces in cities, such as dark-colored cars, buildings, windows, glass panes, black benches and gravestones, are perceived as suitable habitats for these animals. Consequently, they carry out sexual behaviors or oviposit on these unsuitable surfaces (see also Eisenbeis, 2006 for the 'polarization captivity effect').

1.1.5 Non-native and invasive species

Urban environments are often associated with the introduction of non-native species (Kowarik, 2008). These include accidental introductions, for example via the ballast water of ships or via railways and other transportation infrastructure, but also deliberate import of ornamental plants and animals (e.g. terrapins, snakes, goldfish, ornamental garden plants) (Blair *et al.*, 1996; Riley *et al.*, 2005; McKinney, 2006, 2008; Matteson *et al.*, 2014; Gaertner *et al.*, 2017). These can become invasive after introduction in the natural environment. Given that the number of such introductions are higher in urban areas, the likelihood of the establishment of an invasive species significantly increases (Riley *et al.*, 2005; Shochat *et al.*, 2010; Gaertner *et al.*, 2017). In the case of plants, the common preference of people to plant non-native cultivar species (or similar native species) homogenizes urban plant communities substantially (but see Losová *et al.*, 2011; and further 1.2.1 for the 'luxury effect'; Hope *et al.*, 2003; Kinzig *et al.*, 2005; Grove *et al.*, 2006; Smallbone *et al.*, 2011; Clarke *et al.*, 2013; Leong *et al.*, 2018). Non-native species often change biotic interactions dramatically, given that they benefit from niche advantages and predator- and competition-release, thereby outcompeting the native community members (Fischer *et al.*, 2012; Gaertner *et al.*, 2017).

1.2 Biotic responses to urbanization

Clearly, via the myriad of different environmental changes they impose, cities generate conditions that can drastically modify biological life via species extinction/rescue (e.g. urban avoiders versus urban

dwellers) and changes in abundances (negative as well as positive), thus impacting species diversity, community composition, and community trait distributions. Similarly, city centers form multifaceted selection gradients impacting phenotypic trait diversity and possibly the evolutionary trajectories of species (Palumbi, 2001; Alberti, 2005, 2015; Donihue & Lambert, 2015; Johnson & Munshi-South, 2017). Given the high number of environmental changes associated with urbanization, different gradients most likely interact synergistically, antagonistically, or additively. Pinpointing out which specific gradient causes the reported changes in community composition or evolutionary trait shifts is in some cases still less straightforward (e.g. Isaksson, 2015; Thompson *et al.*, 2016; Tüzün *et al.*, 2017a,b), whereas in others more clear (e.g. Zhang *et al.*, 2004; Cheptou *et al.*, 2008; Zipper *et al.*, 2016; Diamond *et al.*, 2017a,b; Harris & Munshi-South, 2017). Additionally assessing the strength of selection in urban areas remains difficult, due to the complexity arising from the interactions among gradients.

First, we will here give an overview of the most commonly associated ecological changes at the level of community composition (1.2.1). Secondly, we report on recent studies showing adaptive and neutral evolution, and the environmental drivers likely shaping these responses (1.2.2).

1.2.1 Urban ecology

Terrestrial ecosystems

Urbanization affects organisms in positive as well as negative ways (e.g.; Rahel, 2002; McKinney, 2006, 2008; Aronson *et al.*, 2008, 2016; Chace & Walsh, 2006; Faeth *et al.*, 2011; Goertzen & Suhling, 2013; Alberti, 2015; Alberti *et al.*, 2017a,b; Ibáñez-Álamo *et al.*, 2017; Lagucki *et al.*, 2017). While most studies on biodiversity report reductions in biodiversity in cities, a few report the opposite (Walker *et al.*, 2009: plants, but due to import of exotic species; Siroh *et al.*, 2015; eusocial bees). Clearly, biodiversity responses are highly taxon-specific and depended on what spatial scale urbanization is assessed or the metric used (McKinney, 2008; Dallimer *et al.*, 2012; Beninde *et al.*, 2016; Lagucki *et al.*, 2017; Piano *et al.*, 2017). Strikingly, researchers found that socioeconomic status impacts biodiversity patterns; wealthier neighborhoods harbor a higher plant, bird, bat and lizard diversity (Clarke *et al.*, 2013; Li & Wilkins, 2014; Ackley *et al.*, 2015), after which they coined this phenomenon the 'luxury effect' (Hope *et al.*, 2003; Kinzig *et al.*, 2005; Grove *et al.*, 2006; Smallbone *et al.*, 2011; Clarke *et al.*, 2013; Leong *et al.*, 2018). This effect is firstly associated with the direct link between economic affluence and vegetative landscaping as rich and wealthy neighborhoods invest in natural landscaping. Consequently, plant coverage and diversity then secondarily impacts animal diversity via resource and habitat provisioning (Faeth *et al.*, 2011). Taking this luxury effect indoors, Leong *et al.*, (2016) reported a significant impact of income on the number of indoor arthropod species; wealthier neighborhoods host higher indoor arthropod diversity (consisting of primarily non-pest species).

While 'the luxury effect' might play a significant role in urban micro-scale biodiversity (i.e. at the level of specific houses and neighborhoods), on larger scales biological communities in cities are in general strongly impacted by 'biotic homogenization' (McKinney, 2006; Niemelä & Kotze, 2009; Brice *et al.*, 2017). Disjunct urban plant and animal communities are compositionally more similar to each other than to their rural counterparts within biogeographic realms (i.e. larger biogeographical regions, e.g. Aronson *et al.*, 2008). This is mainly attributed to habitat homogenization, the higher rate of introductions of ornamental or non-native species (due to human activity, accidental and deliberate; Rahel, 2002; Faeth *et al.*, 2011; Gaertner *et al.*, 2017), the loss and decline of native species (biodiversity loss), and environmental disturbances that feed back to both processes (Brice *et al.*, 2017). Habitat homogenization in urban areas in North-America and Europe (i.e. all characterized by similar urban landscapes such as urban districts, golf courses, canals, and warm-water reservoirs) are predominantly harboring cosmopolitan and generalist bird, arthropod, and plant species, while endemic species are lost (Blair, 2001; McKinney, 2006; Devictor *et al.*, 2007). Early on, urban ecologists often categorized species according to their trait characteristics into urban avoiders, urban adapters, and urban exploiters (Goldstein *et al.*, 1986; Blair & Launer, 1997; Germaine & Wakeling, 2001; but see Fischer *et al.*, 2014 for the use of urban avoiders, urban utilizers, and urban dwellers). Urban avoiders are specialist species, disturbance-sensitive, mostly rare, and with low reproductive investment (e.g. ground-nesting birds or late-successional and wetland plants; Whitcomb *et al.*, 1981; Stein *et al.*, 2000). Urban adapters are more generalist species that can live both in urban and natural environments, and are often edge-associated and early successional. They are able to acclimatize or adapt to urban environments. Well-known examples are rabbits, moles, foxes, birds (e.g. blackbirds, finches, and rock pigeons), Odonata, water birds, and weedy plants. For example, cities and residential areas often offer stable food resources or specific roost places similar to the ones found in their natural habitat (Taylor *et al.*, 2003; White *et al.*, 2005; Faeth *et al.*, 2011), impacting the density of bird species such as rock pigeons (*Columbus livia*; which nests on cliffs). Importantly, the availability of urban green space is often positively correlated with the diversity of urban adapters (White *et al.*, 2005). Urban exploiters, or synanthropes (Johnston, 2001; El-Sabaawi, 2018), are highly dependent on anthropogenic resources and strongly associated with urban settings (Blair, 2001). Urban exploiters are adapted to city environments and highly disturbed habitats (McKinney, 2002) and often invasive or pest species (e.g. brown rats, cockroaches, and the house mouse in Europe; Sorace, 2001; Gaertner *et al.*, 2017). Due to predator- and competitive-release, they often show high population densities in cities (Gering & Blair, 1999; Marzluff, 2001; D'Antonio & Meyerson, 2002; Shea & Chesson, 2002; Sorace, 2001). In plant communities, urban exploiters comprise annual plants and grasses, wind-dispersed weeds, and early colonizers that can grow in small soil patches between stones and pavements (Kowarik, 2008). High habitat fragmentation or low habitat quality (reduced vegetated areas) can impact plant pollinator communities negatively (via decreased species richness and abundance; Ahrné *et al.*, 2009; Hernandez *et al.*, 2009; Bates *et al.*, 2011; Matteson *et al.*, 2014; but see McFrederick & LeBuhn, 2006; Fetridge

et al., 2008; Kearns & Oliveras, 2009; Baldock *et al.*, 2015; Hall *et al.*, 2016; on the importance of green space and vegetated areas, and recent pollinator conservation strategies) ultimately leading to reduced pollination-mediated reproduction in flowering plants (Potts *et al.*, 2010).

Given that species and individuals differ in their ability to tolerate environmental disturbances, the importance of including functional traits in community ecology is highly relevant in the framework of urban ecology (Martinson & Raupp, 2013; Merckx *et al.*, 2018). For example, in Flanders, the study region examined in this thesis (see 3.1), Piano *et al.* (2017) reported a shift in urban carabid beetle communities. Urban communities were characterized by a higher occurrence of thermophilic and dispersive species. This was a result of a higher number of heat-tolerant species, a decreased richness in species with low thermal preference, and a loss of almost all low-dispersal species in the most urban localities. These results are in line with environmental filtering via increased temperatures and fragmentation, which are two important drivers of community assembly in urban areas (McKinney, 2002; Concepción *et al.*, 2015; Parris, 2016; Hung *et al.*, 2017; Piano *et al.*, 2017). For example, in that same region, a recent publication by Merckx *et al.*, (2018), reported on an overarching analysis of community body size shifts across ten terrestrial and aquatic taxa in Flanders. Body size is a key trait affecting an organisms fitness, given its close link with other life-history parameters (e.g. fecundity, competitive strength, predation efficiency, dispersal capacity). As expected, urbanization in Flanders affected body size in the majority of communities, but the direction of community body size change (increase vs. decrease) was strongly depending on the dispersal mode of the taxa. Taxa with low dispersal capacity showed strong body size reductions, most likely as a result of environmental filtering driven by the urban heat island effect (Merckx *et al.*, 2018). Three notable exceptions were taxon groups including moths, butterflies, and grasshoppers and crickets. These taxa showed an increase in community body size with increasing levels of urbanization, suggested to arise from a more stringent effect of habitat fragmentation in urban cores on these actively dispersing organisms. Increased body size in highly fragmented urban patches supports longer dispersal distances, which increases the chances to mate and feed in these taxa.

Lastly, due to the UHI effect, which can shift the start of the growing season in cities substantially, thermal windows for biological growth and development advance. This consequently leads to parallel shifts in the onset of phenological processes such as growth (and associated with that the spatial and temporal shifts in abundances of individuals), mating, and migration in urban bird, plant, and arthropod communities (White *et al.*, 2002; Zhang *et al.*, 2004; Neil & Wu, 2006; Neil *et al.*, 2010; Møller *et al.*, 2015; Seress *et al.*, 2018). For example, the onset of flowering time in urban plants advances on a global scale (Neil & Wu, 2006), likely affecting pollinator dynamics (Hennig & Ghazoul, 2011). Similarly in animals, phenological processes associated with mating and sexual reproduction start sooner. For example, birds sing earlier and more prolonged in urban areas (Møller *et al.*, 2015), with differences in singing duration periods between paired urban and rural populations (along a

latitudinal gradient) as large as differences between populations across southern and northern Europe. Such advances in phenology can have consequences for trophic interactions; while urban birds advanced the start of the breeding season, caterpillar biomass did not follow a synchronized early peak, leading to smaller clutch sizes and higher nestling mortality due to starvation in urban great tits (Seress *et al.*, 2018). Consequently, fewer offspring was reared to fledgling age and fledglings had significantly lower body mass.

Time for a dive: urbanization and its impact on ecological dynamics in freshwater ponds – some highlights

Substantial information on the ecological relevance for biodiversity conservation of 'unusual' urban freshwater habitats such as storm water management facilities is present (Olding *et al.*, 2002; Scher & Thiery, 2005; Foltz & Dodson, 2009; Brand & Snodgrass 2010; McCarthy & Lathrop, 2011; Tixier *et al.*, 2011; Hamer, 2012; Moore & Hunt, 2012; Schaeffer 2012). Small ponds and shallow lakes, with a clear biodiversity and socio-cultural value, received considerably less attention in urban environmental studies (but see Riradevall & Cambra, 1994; Chester & Robson, 2013; Hassall *et al.*, 2014; Thornhill *et al.*, 2017).

Urban ponds are often impacted by various levels of environmental change in their local surroundings, which makes uniform biotic responses to urbanization potentially difficult to confirm. The most predominant ecological responses in urban ponds, that are so far reported on, are mostly linked to isolation, pollution (road salts, eutrophication, metal contamination), mismanagement (removal of vegetation), and morphometric characteristics (van Meter *et al.*, 2011; Hassall, 2014; Hill *et al.*, 2015, 2017; Blicharska *et al.*, 2016; Suski *et al.*, 2018). Additionally, the introduction of non-native (e.g. fish and turtles) and ornamental plant species impact freshwater biodiversity in urban areas (Copp *et al.*, 2005; Hassall, 2014). The majority of studies include amphibians, Odonata (larval and adult stages), and macro-invertebrate assemblages, whereas limited reports on zooplankton or algae communities are present (i.e. in a specific urban context, but see Olding *et al.*, 2002; van Meter *et al.*, 2011; Suski *et al.*, 2018 for more experimental approaches). I will here present the main findings on ecological responses to urbanization in freshwater ponds.

Aquatic vegetation is impacted by eutrophication and the associated occurrence of cyanobacteria, mismanagement of the pond and its surroundings (e.g. removal of vegetation in the pond, reduced vegetated buffer zone around the pond; Noble & Hassall, 2015; Blicharska *et al.*, 2016), and direct consequences of morphometric characteristics. Urban ponds have a simple morphology (rectangles or ovals) and are often characterized by unnatural features such as steep hard-engineered bank angles (Hassall, 2014; Thornhill *et al.*, 2017). These reduce the settlement of aquatic emergent vegetation due to limited soil and sediment build-up. When reviewing literature, it is clear that this removal and reduction of aquatic vegetation is the most common driver of declines in damselfly, dragonfly, amphibian, macro-invertebrate and zooplankton diversity and abundances (Hassall, 2014;

Noble & Hassall, 2015; Blicharska *et al.*, 2016; Thornhill *et al.*, 2017, 2018; Prescott & Eason, 2018). This indicates the intrinsically crucial value of aquatic vegetation as subhabitat providing shelter, food, and mating opportunities. For example, Odonata α-diversity and community composition is primarily affected by fine-scale urbanization as a consequence of absence of vegetation due to intensive management rather than abiotic stressors per se (Jeanmougin *et al.*, 2014; Villalobos-Jimenéz *et al.*, 2016 but see further; Prescott & Eason, 2018, but only effects found in damselflies, not dragonflies). Effects on warming-associated shifts in phenology are only limitedly studied in urban ponds. Villalobos-Jimenéz & Hassall (2017) suggested a shift in phenology, but only at the community level (mean advance in P95 of 4.13 days in urban areas), and concluded the possible effects of UHIs on phenology negligible. The 2016 review of Villalobos-Jimenéz *et al.* (and references therein) did confirm a biodiversity loss due to the concentration of pollutants present in the water, even scaling up to pollutants being present in some more presumably tolerant species (e.g. *Aeshna juncea* and *Platycnemis pennipes*). Rubo & Kiesecker (2005) showed negative impacts of urbanization on breeding distributions of amphibians linked to hydroperiod, habitat connectivity, the size and presence of riparian buffer zones, and the area of forested patches around the pond. Amphibia assemblages (diversity, abundance) show similar responses to pond morphology (i.e. especially depth of the water column and hydroperiod) and vegetation removal as observed in Odonata (e.g. Georgiev *et al.*, 2009; Hamer & Parris, 2011; Hamer *et al.*, 2012). Yet, responses can be extremely species specific (Hamer & Donell, 2008; Hamer *et al.*, 2012). In their 2011 study, Hamer & Parris (2011) found species richness to decrease in ponds with high human resident densities and conductivity, high shading and abundance of predatory fish, whereas aquatic vegetation positively affected community composition. Skelly *et al.*, (2007, 2014), however, showed urbanization alters disease infection dynamics, intersex traits, and deformation in wild amphibian communities. Macro-invertebrate assemblages show reduced species diversity and abundances in urban ponds and pools across Europe (Hassall, 2014; Hill *et al.*, 2015, 2017; Cook *et al.*, 2018). Rich macro-invertebrate assemblages are mostly found in ponds with floating vegetation and complex emergent macrophyte compositions, low nutrient concentrations, and low levels of urban land in their proximity (Hill, 2016, PhD thesis; Thornhill *et al.*, 2017). The most impoverished communities are found in highly urbanized settings. Nevertheless the empirical results are often mixed (e.g. see Hill, 2016, PhD thesis). As expected, homogenization of these communities is not as clearly present compared to observations in terrestrial habitats (Olden, 2006; Hill *et al.*, 2018a), with some studies even indicating higher biodiversity of macro-invertebrates in urban ponds (Hill, 2016, PhD thesis). These effects are however often related to the type of ponds (and pond size; Hill *et al.*, 2015) studied (e.g. floodplain ponds versus park and urban ponds in Hill *et al.*, 2015 and Hill, 2016, Phd Thesis), the scale at which urbanization is assessed (local vs. regional; Hassall, 2014; Thornhill *et al.*, 2017), and the scale at which diversity is assessed (alpha vs. beta diversity). Zooplankton communities show a decrease in average community body size with increasing urbanization partially explained by species turnover from large cladocerans to smaller chydorids (Gianuca *et al.*, 2018; but

see also Chapter 5 of this PhD thesis). While not studied, frequent management and complete water level reductions in ponds could affect sediment build-up and thus the formation of resting egg banks, which is a common feature of most zooplankton species providing seasonal population reestablishment (De Meester *et al.*, 2005). Although they are most strongly linked with biological water quality, habitat value, and ecosystem functions, algae communities are rarely studied in urban freshwater ponds (but see Olding *et al.*, 2002; Peretyatko *et al.*, 2006, 2007, 2009 , 2010; Castilla *et al.*, 2015; and Waajen *et al.*, 2016, for a biomanipulation context). Waajen *et al.* (2014) reported eutrophication-associated increases in cyanobacteria blooms in urban ponds in the Netherlands. In general, morphometric characteristics, such as those typical of many urban water bodies, can play a role in influencing phytoplankton-community composition. These include mixing patterns (Trimbee & Harris, 1984), flooding (Van den Brink *et al.* 1992), water level control and fluctuations (Garcia de Emiliani 1997), high-wind events (Carrick *et al.* 1993), and changes in flushing rate (Bailey-Watts *et al.* 1990). However, it is not known to what extent these physical and morphometric factors are important in modifying the trophic control of phytoplankton-community composition in urban ponds specifically.

It is clear that due to the intrinsic complexity of pond systems and their origin (e.g. European ponds are often historical remnants in urban cores, whereas ponds in suburban and urban regions of the United States are constructed for storm water retention and flood control), uniform responses are limitedly reported on. Some (mesocosm) experiments testing the effects of, for example, road salts, metal pollution, and specific management strategies (e.g. the use of Aquashade) however all confirm detrimental impacts on different components of the aquatic food web, with declines in phytoplankton, zooplankton, macroinvertebrate densities and richness (van Meter *et al.*, 2011; Falfushinska *et al.*, 2015; Ogonowski *et al.*, 2016; Suki *et al.*, 2018). Indirect effects of such stressors are often more subtle. For example salinization reduces zooplankton densities, whereas it increases the development rate of tadpoles, most likely via an indirect effect of reduced top-down control of zooplankton on algae and thus a higher food availability for tadpoles (van Meter *et al.*, 2011). Interestingly, as mentioned before, research on light pollution recently showed surfaces that polarize light to be ecological traps for polarotactic species, such as for example Odonata (Horváth *et al.*, 2009 and references therein). Also noise pollution affects predation efficiency of Odonata (Villalobos-Jimenéz *et al.*, 2017).

In conclusion, freshwater biology in urban areas would benefit from integrated mesocosm exposure experiments, transplant experiments, and regional long-term monitoring of urban ponds to increase our understanding of how urbanization impacts lentic freshwater communities. Citizen Science projects could assist the latter goal (e.g. FreshwaterWatch, Castillo *et al.*, 2015; and the recently set up Urban Algae- Ecological Status and the Perception of Ecosystem Services of Urban Ponds by Sonia Herrero Ortega and Cleo N. Stratmann for FreshProject). A recently developed app for smartphones (Bloomin'Algae; developed by the Centre for Ecology and Hydrology, Scotland, with input from the Scottish Environment Protection Agency, Public Health England and Health Protection Scotland)

allows people to upload pictures of algae blooms, after which these are identified by the app. Information on location, algae bloom type, and timing of occurrence are afterwards stored to build up a data base across Europe, which can be freely used by researchers.

1.2.2 Urban evolution: Darwin taking the subway

Evolution is a change in allele frequencies across generations in a population, driven by different phenomena such as mutation, genetic drift, changes in gene flow, meiotic drive, and/or natural and sexual selection (Hartl & Clark, 2007). Given the novel selective landscape cities impose, repeated reports of adaptive (in response to selection) urban evolution have been published over the past five to ten years (see reviews by Donihue & Lambert, 2015, and Johnson & Munshi-South, 2017). Yet also neutral evolution can occur as a result of landscape features of cities. Urban areas are characterized by habitat fragmentation, reductions in size of remnant patches (thus often resulting in small population size), and, for some species, an unsuitable matrix between habitats. These potentially result in lower genetic diversity within populations and reduced gene flow between population, after which evolution could occur upon strong selection, or elsewise via genetic drift. Genetic drift is the process by which different alleles in different populations are stochastically fixated (Kimura, 1991), thus increasing genetic differentiation among populations. We here highlight some of the most remarkable urban evolutionary changes observed and their most probable drivers.

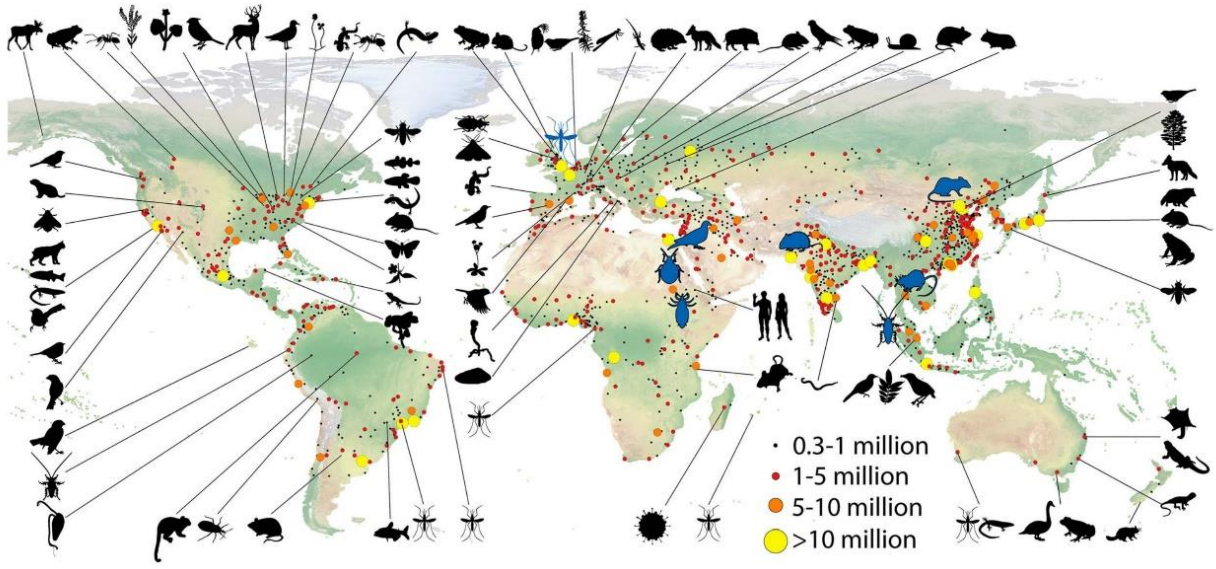

Figure 2. Signatures of urban evolution have been reported now for large number of species across the globe, recently reviewed in Johnson & Munshi-South (2017). Urban evolution includes not only evolution driven by selection, but also neutral genetic differentiation occurring due to mutation, genetic drift, and changes in gene flow. From Johnson & Munshi-South (2017).

Habitat fragmentation and isolation

Genetic drift and founder events (neutral evolution)

A clear example of urbanization driving landscape genetics and neutral evolution was reported in Munshi-South & Kharchenko (2010). Based on a spatial landscape-resistance analysis, they found that high fragmentation and habitat isolation, in combination with little amounts of green corridors between populations, caused a strong decrease in genetic diversity and increase in genetic divergence among New York white-footed mice (*Peromyscus leucopus*) populations. Genetic differentiation among mice populations that were located only a few kilometers from each other is reported to be more significant compared to differentiation among populations that diverged earlier in history when large landmasses in that region got isolated (e.g. Queens, Bronx, Manhattan). Similar observations have been made on the European continent (Gortat *et al.*, 2015; Wilson *et al.*, 2015) in rodents species with a comparable ecological function. The impacts of urban landscape structure on the level of disturbance in gene flow patterns and the occurrence of genetic drift often strongly depend on the dispersal capacity of organisms, the spatial radius of their resource acquisition, and their population size in remnant, often smaller, habitat patches. For example, *Peromyscus leucopus* can establish relatively large population sizes even in small habitat patches, reducing the loss of genetic diversity or heterozygosity as a consequence of drift or genetic bottle necks, respectively (Munshi-South & Karchenko, 2010). Birds, relatively good dispersers throughout most landscapes, suffer less from urbanization-driven losses in genetic diversity as a consequence of reduced gene flow (Björklund *et al.*, 2010; Vangestel *et al.*, 2012; Unfried *et al.*, 2013; Morinha *et al.*, 2016; but see Delaney *et al.*, 2013). In comparison, increasing urbanization negatively impacts butterfly genetic diversity and population size due to limited among-patch dispersal (Rochat *et al.*, 2017). Salamanders, which often depend on green and blue corridors for dispersal, exhibit low genetic diversity and larger genetic population structuring and differentiation in urban compared to rural populations in New York, Montreal, and Oviedo (Spain) as observed for some rodent species (Noël *et al.*, 2007; Munshi-South *et al.*, 2013; Lourenço *et al.*, 2017; but see Munshi-South & Karchenko, 2010 for *Peromyscus leucopus* genetic diversity, and Battles *et al.*, 2018, for Anolis lizards). Cities with sufficient green corridors show limited reductions in gene flow for some butterfly, tree (insect-mediate dispersal), and rodents (Leidner & Haddad, 2010; Goldingay *et al.*, 2013; Noreen *et al.*, 2016), thus confirming the value of such migratory corridors for genetic population structure.

Urban populations are argued to often be newly established by immigration from the peripheral rural areas. This might result in lower genetic diversity or among population genetic differentiation due to the fact that edge populations are often only a small fraction of the core population, increasing the chances of genetic drift and genetic expansion load (i.e. accumulation of deleterious alleles at the range expansion front due to gene surfing, which results from high genetic drift due to low population

densities, in combination with high growth rates; Edmonds *et al.*, 2004; Klopfstein *et al.*, 2006; Peischl *et al.*, 2013). Specific founder events are studied in depth for European blackbird populations (Evans *et al.*, 2009), owls (Mueller *et al.*, 2018), and the recently shown genetic differentiation between surface and underground subway populations of the house mosquito (*Culex pipiens*) in London (UK) and New York (USA) result from the same dynamics, even leading to reproductive isolation and the formation of a new subspecies (Byrne & Nichols, 1999; Kent *et al.*, 2007; Kothera *et al.*, 2010; Kading, 2012).Yet, the increase in genetic differentiation or expansion load as a consequence of founder events in urban populations will strongly depend on the strength of the environmental gradient (rural-to-urban direction), the expansion rate (lower expansion rates allow genetic rescue from the core/rural populations; Gilbert *et al.*, 2017), the population size of the founder cohort, and the number of sequential founder events. Local (mal)adaptation additionally shapes the speed of range expansions and consequently the level of genetic differentiation and accumulation of expansion loads across gradients (Phillips *et al.*, 2010; Gilbert *et al.*, 2017). While studied profoundly along latitudinal gradients, urbanization-associated range expansion dynamics (genetic drift, allele surfing, expansion load, spatial sorting) are so far studied limitedly.

Dispersal, and mobility (adaptive evolution)

Habitat fragmentation and isolation can mediate adaptive evolution of dispersal and dispersal-associated traits (Saaistamoinen *et al.*, 2018). Urban populations of actively dispersing organisms often show differentiation for traits linked to dispersal capacity or locomotory functions, most likely in response to fragmentation (LaPoint *et al.*, 2015, Cheptou *et al.*, 2008; Dubois & Cheptou, 2017; Tüzün *et al.*, 2017c). Evolved changes in dispersal capacity or strategy can increases their mobility in fragmented landscapes and mobility/survival on manmade surfaces commonly found in cities (Rochat *et al.*, 2017; Winchell *et al.*, 2018). In addition, such adaptations might increase the realized connectivity between populations, which promotes gene flow and genetic mixing (LaPoint *et al.*, 2015; Cote *et al.*, 2017; Rochat *et al.*, 2017). Interestingly, for some organisms, fragmentation-driven selection along rural-urban transects might parallel processes found along range-expanding core-edge populations (see higher), resulting in spatial sorting (Phillips *et al.*, 2010; Shine *et al.*, 2011; Saaistamoinen *et al.*, 2018). Additionally, selection for dispersal related traits in urban populations is strikingly similar to shifts in community-level trait distributions. For example, Merckx *et al.*, (2018) found communities of Orthopterans, macro-moths, and butterflies, for which a positive covariation between size and dispersal capacity was found, to be characterized by a higher average community body size in urban compared to rural communities. Larger animals disperse further and have higher chances of mating and food intake, thus indicating a larger body size in active dispersers is regarded beneficial in fragmented urban landscapes.

Extensive studies on Anolis lizards (*Anolis cristatellus*) in Puerto Rico show urban populations have evolved a higher number of toe lamella and longer limb length, which is hypothesized to increase their mobility on concrete, plastic, metal, or glass (Winchell *et al.*, 2016; 2018). The latter was confirmed by performance assays testing sprint speed on three different surfaces (bark, metal, and painted concrete), set up under different inclination angles (0°, 37°, 60°). Sprint speed indeed was higher in animals having longer limbs, larger toepads, and a higher number of toe lamellae. While, overall, sprint speed decreased on the two unnatural surfaces, urban lizards still showed a significantly higher sprint speed on all three surfaces compared to rural lizards, but only at low inclination angels (Winchell *et al.*, 2018). They additionally showed significant reductions in the number of stops during sprinting in urban compared to rural populations. Combined, the results indicate selection in cities favors animals with longer limbs and higher number of toe lamella, which increases their movement speed on unnatural and natural surfaces. In addition selection to increase horizontal dispersal speed (e.g. on the ground or on fences), might be larger than selection for increased sprint speed on inclined perches.

Some grasshoppers (*Chorthippus brunneus*) and butterflies (*Pieris rapae*) have larger wings in urban populations (San Martin y Gomez & Van Dyck, 2012; Schoville *et al.*, 2013), and Tüzün *et al.* (2017c) recently reported urban damselflies to be characterized by higher flight performance compared to rural ones, increasing their ability to colonize new patches, mate, and find new food sources. In the latter study, flight performance was however tested on field-caught animals. The evolutionary background of such fragmentation-driven changes in dispersal capacity is thus not entirely clear.

While actively dispersing animals thus show increased dispersal via locomotory performance, the opposite pattern is reported for plants. Cheptou *et al.*, (2008) reported urban holy hawksbeard (*Crepis sancta*) populations to be characterized by a higher production of non-dispersive seed types compared to rural populations. Reduced dispersal in this species limits non-successful germination on unsuitable surfaces such as pavements and roads (Cheptou *et al.*, 2008; Dubois *et al.*, 2017).

Human presence and food waste

Human activity and diet choices generate large amounts of food litter with high fat and sugar concentrations. Outlier loci analyses on white-footed mice in New York showed 19 candidate genes under selection in urban populations (Harris *et al.*, 2015; Harris & Munshi-South, 2017). Remarkably, the majority of genes were linked to fat and sugar metabolic pathways, thus indicating fat- and sugar-rich food waste in cities might impact the evolution of metabolic pathways. House finches (*Carpodacus mexicanus*) show a change in beak morphology to increase bite force as an adaptation to consume sunflower seeds of bird feeders in urban areas (Badyaev *et al.*, 2008). Especially mammal species and birds show evolutionary divergence linked to urban food waste or diet type and the presence of humans (and associated with that noise). This may also include evolved behavioral changes (Evans *et al.*, 2010; Coogan *et al.*, 2018; but see Miranda *et al.*, 2013 for neophobic behavior in blackbirds). Urban

populations often evolved a higher foraging activity or boldness, even to the extent that individuals show a distinct pro-active personality linked to territorial aggressiveness (Evans *et al.*, 2010; Hardman & Dalesman, 2018) which increases their food gathering, and thus can be regarded adaptive when constantly exposed to human presence or high intraspecific competition. Recent advances in genomic technologies have confirmed common blackbirds (*Turdus merula*) in urban areas have diverged at a specific gene (the SERT gene) linked to harm-avoidance behavior (Garroway & Sheldon, 2013; Mueller *et al.*, 2013). Urban populations are characterized by a lower frequency of the SERT allele compared to rural populations. Similar patterns were found in dopamine receptor genes in black swans (DRD4), for which allelic variants in cities are associated with a decreased wariness to humans (van Dongen *et al.*, 2015; see also Van Donselaar *et al.*, 2018 for behavioral plasticity in black-capped Chickadees (*Poecile atricapillus*) in response to human presence, novel objects, and anthropogenic noise). Urban conditions (high food availability) and reduced shyness in the animals itself, in combination with longer growing seasons in urban areas due to the urban heat island effect, most likely jointly drive the early onset of gonad production (extending the breeding season) and, additionally, reduced migratory behavior in European blackbirds (*Turdus merula*; Partecke & Gwinner, 2007). Combined, they result in higher, presumably adaptive, sedentariness (early reproduction and accessibility to good food sources) in urban populations (but see Herrera-Dueñas *et al.*, 2017).

Overall stress tolerance and chemical pollution

While evidence is still limited for most invertebrate species, some bird and mice studies indicate evolved higher overall stress tolerance and immunological responses in urban populations compared to rural populations (Partecke *et al.*, 2006; Harris *et al.*, 2013, 2015; Costantini *et al.*, 2014; Harris & Munshi-South, 2017). Based on genome-wide single-nucleotide polymorphism (SNP) analyses, Harris *et al.* (2013, 2015) found candidate genes under selection linked to xenobiotic metabolic and immunological pathways in urban white-footed mice. Urban birds show reduced levels of corticosterone stress responses (assessed via common garden rearing experiments), and general lower levels of overall oxidative stress in their blood (Partecke *et al.*, 2006). When reared under common garden settings, pesticide tolerance is higher in damselflies from city populations compared to rural populations (Tüzün *et al.*, 2015). Populations exposed to high contaminant levels possibly evolve lower vulnerability to these contaminants (e.g. Partecke *et al.*, 2006 and suggested by Medina *et al.*, 2007 and references therein), yet, empirical evidence on urban populations is so far too limited to make uniform conclusions on such local adaptation to increased pollution via low vulnerability and alternative stress coping mechanism (Isaksson, 2015).

1.3 Global change and urban planning

Cities are often termed ‘accidental experimental macrocosms’. They already integrate a multitude of disturbance gradients of which many, if not all (e.g. warming, pollution, habitat removal and

fragmentation, introduction of non-native species), are globally expected to reach similar levels in the future due to climate warming and global change (UN, 2014). Therefore, urban centers are regarded valuable research sites; they enable us to study and understand how organisms might respond to future anthropogenic environmental changes. This 'space-for-time' approach (Blois *et al.*, 2013; Janssens *et al.*, 2014; Stoks *et al.*, 2014), suggests spatial gradients can be a tool to study temporal dynamics expected to occur in the future. Particularly with regard to temperature this might be relevant. Due to the UHI effect, urban areas currently already experience temperature increases that are expected to be reached globally under the RCP8.5 climate change scenario (+ 4 °C; UN, 2014). A critical note here, however is, that thermal responses to increasing urbanization may not be merely projected to be the default response to future climate warming, as other disturbances (eutrophication, fragmentation, pollution, changed biotic interactions) interactively shape the outcome of biotic responses, also in cities (Ramalho & Hobbs, 2012; Galic *et al.*, 2018). Climate change and urbanization can, moreover, jointly impact biotic responses (e.g. freshwater communities: Radinger *et al.*, 2016; butterflies: Diamond *et al.*, 2014).

Urban ecology and evolution have the potential to greatly improve policy making regarding nature conservation and urban development strategies. This not only includes the conservation of biodiversity. A renewed integrated, nature-based educational model, and the integration of citizens in science projects (e.g. data acquisition via Citizen Science) has been proposed by numerous scientists (Lepczyk, 2005; Silvertown, 2009; Cartwright *et al.*, 2013; Cosentino *et al.*, 2014; Westgate *et al.*, 2015; Border *et al.*, 2017). This ultimately can result in science and nature education starting in our own backyards, garden parks, school ponds, urban wetlands, and city parks. Bringing human beings, who live in cities and suburban areas, back to nature via nature-educational efforts might strongly impact policy making, urban planning, and biodiversity conservation choices now and in the future (Russ & Krasny, 2017). Currently humans, especially those residing in industrial and urban areas suffer from what is defined as the 'shifting baseline syndrome' (SBS, Soga & Gaston, 2018). This phenomenon describes the gradual change in the accepted norms for the state of natural environments due to the absence of past information or lacking experiences of past natural conditions. The 'Pigeon Paradox' can be interestingly linked to this duality; biodiversity conservation currently does not suffices, yet people will more likely take conservation action when directly exposed to nature. This implies, as people increasingly inhabit urban areas and are being exposed to this 'urban nature', biodiversity conservation in the future might mainly depend on people's interaction with urban biodiversity. Given the shifting baseline syndrome and the current impoverished state of urban biodiversity, citizen science and scientific investment in clear cut, popularized summaries of urban ecological and evolutionary studies, published in popularizing science journals or on blogs increase the visibility of the domains and can contribute to policy making and human awareness for the current state of natural biodiversity in cities.

2 Research aims

Evidence of ecological and evolutionary changes in species communities and populations as a consequence of urbanization is growing rapidly, but the majority of studies focus on birds, mammals, and terrestrial arthropods and plants (e.g. see McKinney, 2006, 2008; Donihue & Lambert, 2015; Parris, 2016; Johnson & Munshi-South, 2017). Freshwater ecosystems, and more specific lentic waterbodies such as ponds and shallow lakes (Fig. 3), in general a neglected habitat type (but see Brönnmark & Hannson, 2002; Oertli *et al.*, 2002; De Meester et al, 2005; Scheffer *et al.*, 2006; Céréghino et al, 2008), have been studied with regard to land cover changes such as agriculture and industrialization (Williams *et al.*, 2004; Biggs *et al.*, 2005; Declerck *et al.*, 2006; Davies *et al.*, 2008; Downing *et al.*, 2008). Yet, they are particularly underrepresented in studies concerning urbanization (Birch & McCaskie, 1999; Chester & Robson 2013; Hassall, 2014; Hill *et al.*, 2015, 2017, 2018a; Blicharska *et al.*, 2016, 2017; Thornhill *et al.*, 2017, 2018).

While urban ponds are often small, they represent an important resource for species conservation given that even in urban areas, local environmental heterogeneity causes high community variability among ponds (through space and time, Céréghino *et al.*, 2014; Pinel-Alloul & Mimouni, 2013). Urbanization-associated environmental changes surrounding ponds can change from city to city, and even differ between ponds in one city. Therefore urbanization possibly does not generate 'similar' habitats across or within cities, as is often the case for terrestrial systems via habitat homogenization (Rahel *et al.*, 2002; Groffman *et al.*, 2014; Hill *et al.*, 2017). Rather, urbanization results in a heterogeneous mix of pond types (e.g. see Chester & Robson, 2013; Hill *et al.*, 2017), which makes them an intriguing study system given that ecological and evolutionary responses in freshwater communities across urban landscapes might substantially differ across cities.

A number of very recent studies do provide strong evidence of ecological and evolutionary changes in urban freshwater communities and populations (see 1.2.1). However information is still limited and scattered. So far it is unclear whether the observed ecological and evolutionary responses to urbanization run in parallel with reports on terrestrial ecosystems or what the specific drivers of the responses are. In order to make valid comparisons (e.g. via meta-analyses) on differences (or similarities) in biotic responses to urbanization in terrestrial versus freshwater ecosystems, more data need to be collected along rural-urban transects including freshwater ponds, pools, and streams. Yet, while local environmental factors might substantially differ among urban ponds, we hypothesize a recurring effect of urbanization on small ponds might be anthropogenic warming. In addition, a crucial difference in how urbanization affects freshwater ecosystems, compared to terrestrial habitats, could be more severe oxygen-limitation due to warming (reduced solubility of oxygen and increased microbial respiration at higher temperatures) and eutrophication (increased oxygen consumption by algae) in these systems (see further).

We set out to test whether we can still detect similar urban signals of phenotypic (genetic or plastic) trait responses in populations and communities across multiple replicated urban ponds in a region (here Flanders, Belgium). The topics listed below represent a number of research gaps in the general field of urban ecology and evolution (in some instances more directly linked to freshwater biology), which I aimed to address in one or more chapters of my dissertation and which are schematically outlined in Figure 3 (numbered circles indicate the four presented research gaps below, which are accordingly addressed in one or more chapters of this thesis).

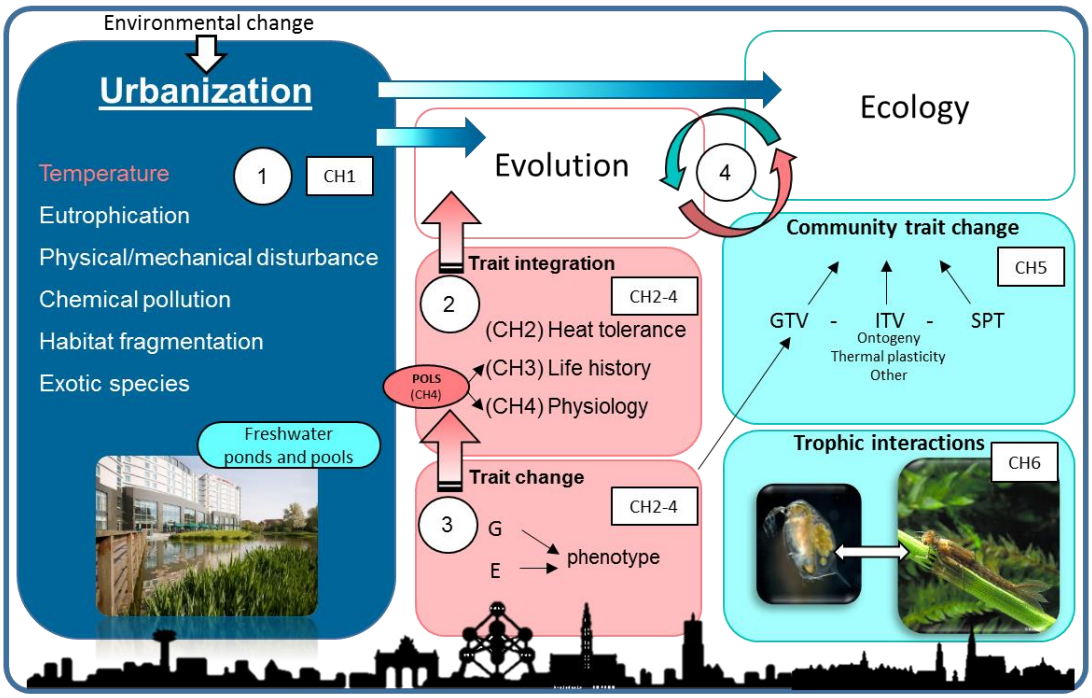

Figure 3. Schematic visualization of the current relevant research gaps in the fields of urban evolution and urban ecology (circles with numbers 1 to 4), addressed in the different chapters of this PhD thesis (Chapters 1 to 6). In general lentic small freshwater ecosystems (e.g. inset picture) are valuable for biodiversity conservation, but so far limitedly studied in cities. We thus focus on freshwater ponds and pools. During my PhD, I aimed at testing whether urbanization impacts the thermal characteristics of ponds and pools (1 – Chapter 1), how this translates into the evolution of multiple traits (and their possible trait covariation patterns) in one species (2 – Chapters 2 to 4), what the relative contribution of genetic differentiation (G; driven by evolution) and phenotypic plasticity (i.e. E, purely driven by the environment) is to the observed phenotypic trait shifts (3 – Chapter 2 to 4), and lastly, whether evolution feeds back to ecological dynamics such as community composition (intra-guild) or trophic interactions (e.g. predator-prey interactions) (4 – Chapter 5 and 6).

2.1 Temperature, heat tolerance, and body size

While a large number of studies show phenological shifts in plant, bird, and arthropod communities due to urbanization-driven warming and its associated growing season alterations (Jochner *et al.*, 2013; Neil *et al.*, 2010; Jochner & Menzel, 2015; Møller *et al.*, 2015; Villalobos-Jimenez & Hassall, 2017; Seress *et al.*, 2018), evolutionary or ecological shifts in functional traits along urbanization-driven temperature gradients is only limitedly studied. Evidence on, for example, on the evolution of heat tolerance or

thermal performance curves (TPCs) in urban populations is extremely rare (heat tolerance: Diamond *et al.*, 2017b for acorn ants, but possibly strong maternal effects; TPCs: McLean *et al.*, 2005 for fungi, and recently Tüzün *et al.*, 2017b for damselflies).

Aquatic ecosystems are regarded to mitigate terrestrial urban heat islands (Völker *et al.*, 2013; Gunawardena & Kershaw, 2016). However, this requires them to be of a substantial size (e.g. lakes or large rivers). Small aquatic ecosystems such as ponds and pools might equally suffer from warming or shifts in other thermal characteristics such as temperature variation patterns, extreme temperature events, or growing season length. So far, no empirical evidence for such urbanization-driven warming of these systems is currently present. Given that the majority of animals inhabiting ponds are ectotherm species, studying the UHI effects in small aquatic systems can be of substantial relevance. Higher temperatures and increased temperature fluctuations not only increase thermal stress (Overgaard *et al.*, 2014; Vasseur *et al.*, 2014; Buckley & Huey, 2016; Pörtner *et al.*, 2017), but also higher the oxygen demands of ectotherm animals. Yet, at the same time, warming decreases the solubility of oxygen in water, thus potentially generating oxygen stress for these organisms (Bozinovic & Pörtner, 2015; Chown & Duffy, 2015; Horne *et al.*, 2015; Pörtner *et al.*, 2017; Hoefnagel *et al.*, 2018).

We hypothesize that it is thus not unlikely that urbanization, via an aquatic urban heat island effect, operating via changes in mean, minimum, and extreme temperatures or via changes in the length of the growing season, causes selection for more warm-adapted and hypoxia-tolerant genotypes and species (Angilletta *et al.*, 2004, 2007: Angilletta, 2009; Overgaard *et al.*, 2014; Vasseur *et al.*, 2014; Chown & Duffy, 2015). Next to heat tolerance, other functional traits, such as life-history traits might evolve in urban populations in response to increased warming (Tüzün *et al.*, 2017b; Daufresne *et al.*, 2009; Ohlberger, 2013). For example, in line with earlier observations in response to climate warming (Deutsch *et al.*, 2008; Gardner *et al.*, 2011; Sheridan & Bickford, 2011; Naya *et al.*, 2017) body size might be an evolutionarily responsive trait in urban settings and warming can result in smaller sized animals (i.e. according to the temperature-size rule, Atkinson, 1994; Angilletta *et al.*, 2004; Angilletta, 2009).

2.2 A multi-trait approach in a multi-city context

2.2.1 Measuring multiple traits

A number of complexities are associated with studying organismal responses to urbanization gradients. Multiple disturbance gradients coincide in cities (Kaye *et al.*, 2006; Grimm *et al.*, 2008a,b; Ramalho & Hobbs, 2012; Parris, 2016; Galic *et al.*, 2018), responses are most likely taxon-specific based on dispersal modes, body size, or life history of organisms (e.g. Merckx *et al.*, 2018), and urbanization-driven changes in the environment often have a specific scale-dependency (San-Martin Y Gomez & Van Dyck, 2012; Kaiser *et al.*, 2016; Leveau *et al.*, 2017). While in general overall signatures of

urbanization in phenotypic and genotypic trait change have been reported (see Alberti, 2005, 2015; Alberti *et al.*, 2017a,b), the majority of urban studies only integrate a limited (mostly single) number of traits. Yet, the intrinsic complexity associated with city environments, as mentioned above, calls for a more mechanistic, integrated, underpinning of observed responses for different taxa, including the joint assessment of life history, behavior, physiology, and immunology, as these might elucidate the potential stressors driving the observed trait changes and traits mostly do not (co)vary independently.

Urbanization is often defined as a 'stressful' environment for many organisms (but see Kark *et al.*, 2007; Driscoll *et al.*, 2009; Aplin *et al.*, 2011; for those species indicative of exploitative adaptations, often commensals, associated with the proliferation of pest species). Numerous studies show how exposure to chemicals, changed diets, or extreme temperatures can change stress physiological responses in plants and animals (Knops *et al.*, 2001; Isaksson, 2010; Costatini, 2014; Janssens *et al.*, 2014; Dinh *et al.*, 2016). Populations exposed to high contaminant levels or other environmental stressors, may evolve lower vulnerability to those stressors (Medina *et al.*, 2007). How urbanization impacts fundamental stress physiological responses (or the relaxation of stress) such as the concentration of energy metabolites (proteins, sugars, fat), the activity of antioxidative repair enzymes, the concentration of stress hormones or heat shock proteins, the level of oxidative damage, or presence of specific detoxification pathways, is only investigated in a limited set of species, mostly birds, mammals, or fish (Partecke *et al.*, 2006; Isaksson, 2010, 2015; Whitehead *et al.*, 2012; Costantini *et al.*, 2014; Reid *et al.*, 2016; Harris & Munshi-South, 2017). Yet, summarized findings of studies reporting on such stress physiological responses indicate specific oxidative stress responses are often multi-directional (no unifying link between increasing urbanization and the level of stress adaptation or acclimatization could be found), most likely due to the strong city- and taxon-specificity of responses, the type of analysis (field observations, common garden experiments, genetic screening) and the limited set of stress-related responses measured. For example, in some cases only antioxidant defense was assessed, or antioxidant damage, but not both (Isaksson, 2010, 2015). An exceptional example is the profound work done on killifish (*Fundulus heteroclitus*), for which researchers reported evolved tolerance to polychlorinated biphenyls via a modification of the aryl hydrocarbon receptor-based signaling pathway (Whitehead *et al.*, 2012; Reid *et al.*, 2016).

A clear gap on urbanization-driven changes in the evolution of stress defenses and energy resources remains for invertebrate species, although they do constitute the majority of biodiversity on Earth. Investigating trait measurements at the physiological level can elucidate mechanistic drivers of other responses observed (e.g. life history or behavior) and understand which disturbance gradients cause the observed changes.

2.2.2 Trait integration: exploring the Pace-of-life Syndrome Hypothesis

A multi-trait approach opens up the possibilities to explore not only the evolution of mean trait values but also trait covariation patterns, as proposed by the Pace-of-life Syndrome Hypothesis (Ricklefs & Wikelski, 2002; Réale *et al.*, 2010; Monceau *et al.*, 2017; Wright *et al.*, in revision). Organisms usually fall at either end of what is defined as the 'pace-of-life axis' (Wikelski *et al.*, 2003; Wiersma *et al.*, 2007; Lodoño *et al.*, 2015). Slow-paced species are characterized by slow developmental rates, large body sizes, low number of juveniles, and high survival, whereas fast-paced species show the opposite set of life-history traits. Pace-of-life can be studied across species, but also across populations. A few studies show urban birds are characterized often, but not always, by a slow pace-of-life due to offspring investment, high parental care, and a reduction in predation pressure (Charmantier *et al.*, 2017, and Sepp *et al.*, 2017).

As an extension, the Pace-of-Life Syndrome (POLS) hypothesis predicts coevolved correlated suites of physiological, behavioral, and immunological traits along that pace-of-life axis in such way that fast-lived species for example, are more active and bold, have a higher metabolism, higher oxidative stress levels, and a reduced investment in immune function (Réale *et al.*, 2010; Careau *et al.*, 2011; Tieleman, 2018). POLS are predicted to not only diverge between species, but also populations and even individuals within populations (Réale *et al.*, 2010; Debecker *et al.*, 2016). Additionally, it is suggested that POLS expression is highly context-dependent and possibly under selection in disturbed and stressful environments (Bell & Sih, 2007; Sih *et al.*, 2012; Snekser *et al.*, 2009; Binder *et al.*, 2016). While pace-of-life in general receives much attention in behavioral ecology (Brodin, 2009), evidence for the covariation of life history, behavior, and physiology in the form of a syndrome is mixed and has been not profoundly tested in the context of natural or anthropogenic disturbance gradients such as urbanization (Damhahn *et al.*, 2018; Montiglio *et al.*, 2018; but see Evans *et al.*, 2010; Miranda *et al.*, 2013; Charmantier *et al.*, 2017; Sprau *et al.*, 2017) and focuses mainly on behavior-life history associations.

2.2.3 Parallel evolution: measuring responses in multiple cities

Additionally, although evidence of urbanization structuring community dynamics across regions in a similar way are prominent (McKinney, 2006, 2008; Devictor *et al.*, 2007; Deguines *et al.*, 2016), the evidence of city environments shaping parallel evolution (i.e. similar evolutionary responses across species) are less evident, partially due to the fact that most urban evolutionary studies focus on one or two cities. To define clear evidence of urban signatures in evolution, multiple cities should be included, preferentially all characterized by sampling distinct rural-to-urban gradients in each city (e.g. Thompson *et al.*, 2016; Diamond *et al.*, 2018a). Such a sampling strategy enables to detect parallel urban evolutionary clines. Including multiple cities within a study region will be a step forward to detect overall urbanization-driven evolutionary trends and/or city-specific responses. For example, Thompson

et al., (2016) reported similar evolutionary evolved decreases in anti-herbivore chemical defense (i.e. cyanogenesis, or the formation of hydrogen cyanide) across four North-American cities in white clover. The observed responses were linked to a decrease in snow cover during winter in urban areas, selecting for increased freezing tolerance in chemically defended plants. The previously mentioned locomotory adaptations in Puerto Rican Anolis lizards also included multiple urban and rural sites across the island (Winchell *et al.*, 2016, 2018). Lastly, Virginia weed in four urban populations in North America show heritable changes towards faster growth, larger size, earlier flowering and greater fitness (Yakub & Tiffin, 2016), compared to rural populations.

2.3 Phenotypic plasticity versus evolutionary trait change

A large number of initial urban evolutionary studies reported phenotypic trait shifts across urbanization gradients (but see recently Alberti *et al.*, 2017b; Table 1 therein for genetic versus phenotypic basis; and Johnson & Munshi-South, 2017). While valuable for our understanding on how urbanization impacts trait distributions, observed trait shifts based on field observations do not disentangle pure evolution from phenotypic acclimatization responses. Phenotypic plasticity (i.e. the capacity of a genotype to produce different phenotypes in different environmental conditions; Via & Lande, 1985), is hypothesized to be crucial for short-term survival but might not buffer populations and individuals from extinction (but see Hendry & Merilä, 2014). While it is not clear whether such plasticity has the potential to accelerate or constrain evolution (Ancel, 2000; Price *et al.*, 2003; Grether, 2005; Ghalambor *et al.*, 2007; Paenke *et al.*, 2007; Lande, 2009; Cameron *et al.*, 2015), it is expected that novel environments with high disturbance levels and inconsistent cues might select for those genotypes with a higher trait 'versatility' (i.e. evolution of phenotypic plasticity; Lande, 2009; Chevin *et al.*, 2010; Gibbin *et al.*, 2017). While this hypothesis has been suggested by many and is highly relevant in the context of, for example urbanization, general empirical evidence is limited (Ghalambor *et al.*, 2007; Chevin *et al.*, 2017; Gibbin *et al.*, 2017; but see recently Diamond *et al.*, 2018b on the evolution of heat tolerance plasticity in urban acorn ants), and supports additionally the presumed importance of adaptation via the evolution of mean traits (Urban *et al.*, 2016; De Meester *et al.*, 2017).

We therefore suggest a more detailed exploration of the relative contribution of evolution, plasticity, and the evolution of plasticity to within-species trait change across rural-urban settings via targeted common garden experiments (e.g. by using multiple common garden experimental treatments linked to relevant urban stressors such as temperature or pollutants) or transplant experiments, and clear-cut partitioning metrics (e.g. as in Stoks *et al.*, 2016; Govaert *et al.*, 2016).

2.4 Urban Eco-evolutionary feedbacks: community context

Ecological and evolutionary time scales are now widely recognized to converge resulting in the emerging research field of 'eco-evolutionary dynamics (Thompson, 1998; Hairston *et al.*, 2005; Fussmann *et al.*, 2007; Urban *et al.*, 2008; Palkovacs & Post, 2009; Ellner *et al.*, 2011; Schoener, 2011; Shefferson & Salguero-Gómez, 2015; De Meester *et al.*, 2016; Hendry, 2017), which seeks to understand how, in space and time, ecological dynamics feed back to evolutionary processes and vice versa. While a large body of studies convincingly show eco-to-evo feedbacks, the opposite part of the eco-evolutionary equation remains mostly studied in proof-of-principle experimental set-ups (Yoshida *et al.*, 2003; Fukami *et al.*, 2007; Matthews *et al.*, 2011, 2016; Rudman & Shluter, 2016; Pantel *et al.*, 2015; Gómez *et al.*, 2016; Brunner *et al.*, 2017) or predicted via theoretical modelling (Loeuille & Leibold, 2008; de Mazancourt *et al.*, 2008; Norberg *et al.*, 2012; Vanoverbeke *et al.*, 2016). While the majority of such proof-of-principle experiments do show that evolutionary trait change impacts population dynamics and composition (e.g. Fukami et al, 2007; Brunner *et al.*, 2017), species interactions (Becks *et al.*, 2011; Friman *et al.*, 2015); community composition (terHorst *et al.*, 2014; Pantel et al;, 2015; Gómez et al;, 2016) and ecosystem characteristics (Harmon *et al.*, 2009; Bassar *et al.*, 2010), evidence for specific feedbacks of evolution to ecological processes in nature is more limited (but see examples on specific 'community genetics': e.g. Hochwender & Fritz, 2004; Bangert *et al.*, 2005, 2006, 2008; Whitham *et al.*, 2006; LeRoy *et al.*, 2006; Johnson, 2008; Barbour *et al.*, 2009; Crutsinger *et al.*, 2014a,b; and additionally Palkovacs & Post, 2009; Bassar *et al.*, 2010; Pantel *et al.*, 2011; Walsh *et al.*, 2012; terHorst *et al.*, 2014; Matthews *et al.*, 2016).

Interestingly, given the speed at which urban development changes the natural environment, and the growing evidence of fast evolution in the city, especially urban and suburban areas could be a key setting to study the ecological consequences of evolution. Alberti *et al.*, (2017b) synthesized how evolution in cities could alter ecological features of city communities and ecosystems, yet, the proposed feedbacks were mostly hypothetical, and in most cases not empirically tested. Also, in urban eco-evolutionary research, it would be worthwhile comparing trait responses at the community level with evolutionary responses observed across populations. While community trait shifts can be a result of species replacements (as a consequence of interspecific trait variation) and changes in abundances of species, they also possibly originate from intraspecific trait variation as a result of phenotypic plasticity or genetic differentiation (evolution). Yet, whether evolutionary trait changes run in parallel with observed trait shifts at the community level is so far still limitedly studied, at least along urbanization gradients. In addition, partitioning the relative contribution of intraspecific and interspecific trait variation to community trait shifts along environmental gradients has been done (Lajoie & Vellend, 2015), isolating the evolutionary intraspecific component of the observed community trait change implies integrating genotypic trait data measured on specific species (e.g. from common garden experiments) with community trait data gathered along the same gradient (based on in situ field

measurements, and preferably including trait and abundance data on all species present in each community along the sampled gradient). Such clear partitioning of evolutionary contributions to community trait change has not been done yet and can be highly relevant in the framework of urban ecology and evolution.

Finally, another clear hiatus in current urban evolutionary and eco-evolutionary studies (in general) is the fact that the majority of research is performed on one focal species, whereas it is probable that multiple species in a community will show evolutionary trait change, which can affect their interaction and community dynamics. A few studies on microbial organisms integrate such multi-species approach (Celiker & Gore, 2014; Fiegna *et al.*, 2015a,b; Low-Décarie *et al.*, 2015), however never in the context of urbanization.

We thus see a threefold gap in eco-evolutionary research: 1) can we detect evo-to-eco feedbacks in communities sampled along an urbanization gradient? And if so, what is the relative contribution of evolution, plasticity, the evolution of plasticity, and species sorting to observed community trait shifts? 2) How do evolutionary trait shifts align with observations at the community level, i.e. does evolutionary trait change within a species parallel community trait shifts? 3) Does multi-species evolution impact ecological interactions, for example resource-consumer dynamics, predator-prey dynamics, or host-parasite interactions?

3 Study system

3.1 Metropole Flanders

The study region of this project is Flanders (Northern part of Belgium; Fig. 4). Flanders has undergone a three-phased urbanization process, including urbanization during the Middle Ages, the Industrial Revolution, and after World War I (Kesteloot, 2003; Tempels *et al.*, 2012). The region has known a substantial increase in urbanization over the past 70 years (Fig. 4; Poelmans & Van Rompaey, 2009; Verbeek & Tempels, 2016). Flanders has a population size of ca. 6.6 million people and comprises a surface area of ca. 13.500 km^2. As a result, Flanders is one of the most densely populated (ca. 490 inhabitants/km^2) and built-up areas in Europe. Urbanization levels and population size are expected to increase to more than 41% and 7 million respectively by 2050 (Poelmans, 2010, PhD dissertation; Fig. 4). Currently, urbanization in this region is defined as urban agglomeration, including polycentric urban areas which are linked via transportation infrastructure and ribbon development (Poelmans, 2010, PhD dissertation).

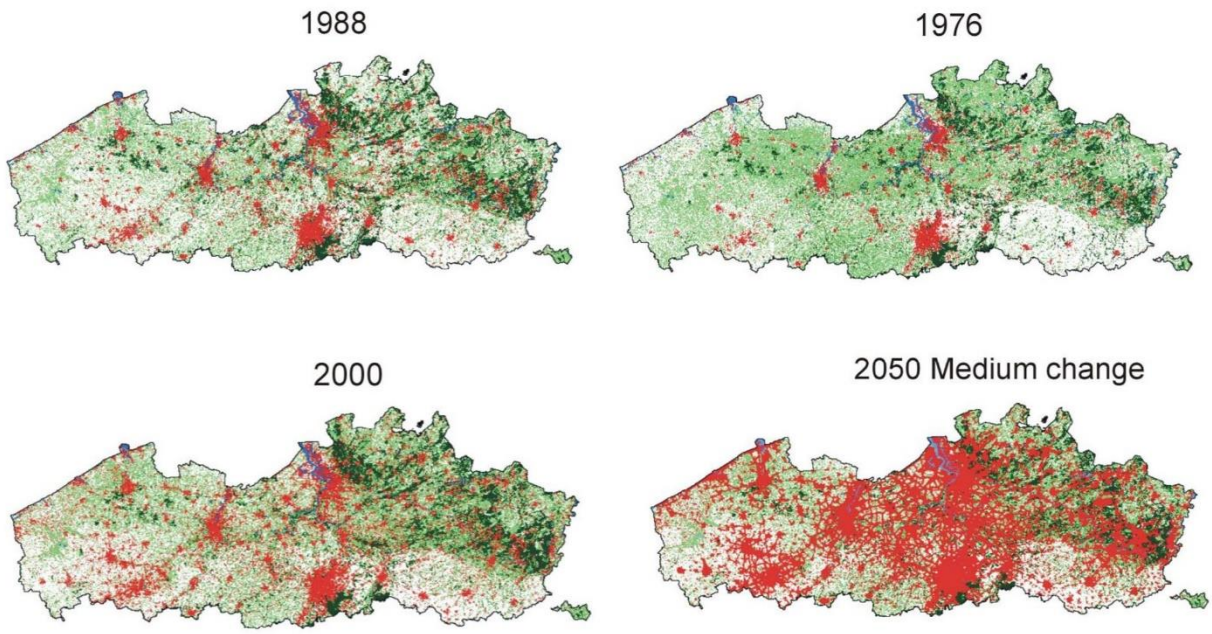

Figure 4. Historical and projected (under a scenario of medium population increase by 2050) land cover changes as a consequence of urbanization in the study region examined in this thesis (Flanders, Belgium; white: arable land, light green: grassland, dark green: forest, blue: water, red: built-up area). From Poelmans (2010, PhD dissertation).

3.2 SPEEDY

My PhD research is part of a larger project, entitled Spatial and Environmental Determinants of Eco-evolutionary Dynamics in urbanized landscapes (SPEEDY; Belspo IAP). The consortium, a collaboration between different international and national universities, gathers research on ecological and evolutionary responses of species to urbanization-mediated anthropogenic disturbances in Flanders (Northern Belgium). SPEEDY aimed at testing how multiple species and organisms groups respond to urbanization, what the relative contributions of evolutionary and ecological processes are, how ecology and evolution might feed back to each other (cfr. eco-evolutionary dynamics; Alberti, 2015; Hendry, 2017), and what the potential underlying mechanisms are of observed community and population responses.

In total five terrestrial (e.g. spiders, birds, butterflies and moths, carabid beetles) and seven aquatic or semi-aquatic (e.g. damselflies, water fleas, ostracods, rotifers) organism groups were sampled and monitored. Via the elaborate interuniversity collaboration, this project succeeded in studying the impact of urbanization in a multi-species context, covering the same urbanization gradients in the same geographical region. The taxa span a broad range of body sizes, dispersal modes (passive to active; short distance to long distance), generation times (short to long), and reproduction modes (sexual to asexual). This enables testing whether urbanization effects on ecology and evolution are

taxon-specific, depending on dispersal mode, and for example different in terrestrial versus aquatic ecosystem.

Speedy applied a hierarchical sampling design, covering a large area of Flanders including the urban areas around Antwerp, Ghent, Brussels, Mechelen, and Leuven (see for example Fig. 1 in Merckx *et al.*, 2018). The sampling design integrated both local (200 x 200 m subplots) and regional (3 x 3 km plots) urbanization. In 27 large 3 x 3 km plots urbanization was assessed based on the percentage Built-up Area (%BA; including buildings, housing, industrial infrastructure, but excluding roads and parking lots; derived from the Large-scale Reference Database and LRDgis maps of the Flemish Geographical Information Agency, using Gis software package ArcView GIS 3.2a, ESRI Inc). Plots were classified as urban (10% BA), semi-urban (5-10% BA), or rural (< 5 or < 3% BA depending on aquatic or terrestrial habitats). Additionally, at the regional level, rural sampling points were characterized by at least 20% of Biologically Valuable Area, in order to avoid sampling in areas with low built-up area but high levels of agriculture. In each plot, three additional subplots were sampled of each local urbanization class (urban, semi-urban, and rural, assessed at the 200 x 200 m resolution). All organisms groups were sampled in the same regional plots, while subplots depended on the habitat specifics and preferences of the focal taxa. With this set-up, the SPEEDY project convincingly advanced towards a more holistic research approach (i.e. scale-dependency, taxon-specificity; Garden *et al.*, 2010; Alberti, 2015; Alberti *et al.*, 2017b: Merckx *et al.*, 2018).

3.3 Ponds and pools

Freshwater ecosystems such as ponds and small lakes have been a relatively neglected habitat for years given that they were regarded simply as smaller versions of large lakes (but see De Meester *et al.*, 2005; Dudgeon *et al.*, 2006; Céréghino et al, 2008; Boix *et al.*, 2012; Hill *et al.*, 2018a,b). Although national and international policy making increased the protection of freshwater habitats, in reality mainly rivers (Dallimer *et al.*, 2012) and larger standing waterbodies (≥ 50 ha) are included (e.g. European Waterframework Directive, WFD; Ramsar convention, Ramsar, 2016; "Priority Habitat" and "Priority Species" site- and species-specific conservation policies in the UK, JNCC, 2012) next to specific small freshwater habitats (e.g. Mediterranean temporary ponds, EU Habitat Directive).

Small ponds and shallow lakes provide essential habitat for numerous animal and plant species, such as macrophytes, zooplankton, macro-invertebrates and fish (De Meester *et al.*, 2005; Oertli *et al.*, 2002). In addition, they are an essential place for feeding, mating, and egg-depositing and crucial migratory stepping stones for many semi-aquatic (e.g. Amphibia and Odonata) and even terrestrial organisms (e.g. water birds) (Fortuna *et al.*, 2006). Given the discrete nature of ponds, and their small catchment area, the strong heterogeneousness and amplitude of environmental characteristics in the proximity of ponds generate diverse local habitat conditions, harboring different and often endemic

species (Biggs, *et al.*, 2005; Davies, 2005; Pinel-Aloull & Mimouni, 2013). As a consequence of increased community dissimilarity, ponds are thus important to conserve regional biodiversity (beta and gamma diversity) (Williams *et al.*, 2004; Hill 2015). They are additionally ubiquitously present in the landscape (Downing *et al.*, 2006), with higher pond density increasing species richness, also in urban environments (UK urban ponds, Gledhill et al 2008). For these reasons pond networks in the landscape (i.e. 'pondscapes'; Boothby, 1997; De Meester *et al.* 2011) support higher taxonomic richness and ecological value (e.g. habitat diversity, migratory stepping stones) compared to larger lakes (Oertli *et al.*, 2002; Downing *et al.*, 2006). It is argued that local monitoring, restoration, or replacement of lost ponds (due to land cover changes such as agriculture or urban development), does not suffice to restore ecological balance. Rather, pondscapes should be the focal unit of integrated pond conservation and management (WFD; Pyke, 2005; Miracli *et al.*, 2010; De Meester *et al.*, 2011; Céréghino *et al.*, 2013; Biggs *et al.*, 2016; Hill *et al.*, 2018b). Next to their high contribution to biological diversity and their ecological importance, small ponds and shallow lakes provide ecosystem functions such as hydrological regulation, nutrient interception (nitrogen and carbon sequestration), and biological water purification and bioremediation via microbial- and vegetation-associated removal of contaminants. Additionally, humans benefit from the presence of blue space in recreational settings, as they are beneficial for human psychological wellbeing (Syme *et al.*, 2001; Ehrlich, 2002; Lee & Maheswaran, 2011; Papayannis, 2011; Hasall, 2014). In combination, the ecological and socio-cultural value of ponds and pondscapes is starting to emerge, advocating a need of the inclusion of ponds in national and international legislation and policy frameworks, as recently urged for (Dudgeon *et al.*, 2006; Hill *et al.*, 2018b).

In this thesis, I focus on zooplankton, and in particular the large-bodied zooplankton species *Daphnia magna* (Fig. 5a). Large zooplankton species, such as *D. magna*, have an important dual role in freshwater food webs and ecosystems. Planktonic food webs in ponds often include four major groups: phytoplankton, the primary producers forming the base of the food web, zooplankton, the primary consumers, macro-invertebrates and planktivorous fish, the secondary consumers, and piscivorous fish, the top-predator of the system (Fig. 5b; Brooks & Dodson, 1965; Jeppesen *et al.*, 1991; Scheffer *et al.*, 1993). Shifts from clear to turbid states can occur in ponds and are the result of an interaction between bottom-up and top-down processes (Fig. 5b). Positive and negative feedback mechanisms between these trophic groups and macropyhtes have been well-studied. Large zooplankton species, for example flea *Daphnia magna*, actively graze down algae, thereby providing 'top-down' control on phytoplankton communities (Jeppesen *et al.*, 1991; Cottenie *et al.*, 2001; Peretyatko *et al.*, 2009). They additionally represent an important food source for macro-invertebrates, such as damselfly and dragonfly larvae, and small planktivorous fish. Large cladocerans thus constitute the energy transfer between trophic levels, but also actively contribute to stable clear-water states. Aquatic vegetation stabilizes this clear-water phase as it provides refuge for large zooplankton, reducing the risk of predation. Importantly, macrophytes limit sediment resuspension (and thus nutrient release) and

actively compete for nutrients and light with algae. Macrophytes release alellopathic substances that prevent algae growth, and thus the build-up of algal biomass. Yet, ponds often range along an oligotrophic to eutrophic nutrient status, the latter rendering them susceptible to a fast turnover to a phytoplankton dominated, turbid, stable state. Eutrophication can either occur via external depositions of nutrients (nitrogen and phosphorous) into the water, but also via 'internal' depositions of nutrients via water bird faeces or sediment resuspension by fish for example (Fig. 5b). Upon eutrophication, competition for nutrients and light between macrophytes and algae increases (i.e. a bottom-up effect). When light conditions are too limited, macrophytes die off. This reduces resource-competition with algae, and additionally leads to a decline in the availability of refugee habitat for zooplankton. Macrophyte reductions can subsequently contribute to oxygen depletion via the microbial decomposition of dead macrophyte biomass, which can render the pond unsuitable for zooplankton, macro-invertebrates, amphibian, and fish due to severe hypoxia. Planktivorous fish are size-selective visual predators and will mainly predate on large zooplankton, which reduces the zooplankton-algae top-down control, further promoting algae densities to increase. Together with the internal eutrophication originating from fish faeces into the water and the resuspension of sediments (due to fish movement and the absence of macrophytes), the pond shifts to a turbid state (Scheffer *et al.*, 1993). In these turbid states, zooplankton communities are mainly characterized by small species such as *Bosmina* sp., *Chydorus* sp., and *Ceriodaphnia* sp., and high algal biomass. When temperatures increase, the risk of a third stable state, dominated by cyanobacteria increases, (Kosten *et al.*, 2012; Huisman *et al.*, 2018) as the majority of blue algae species have higher thermal optima and can actively regulate their position in the water column. Cyanobacteria can thus monopolize nutrients and lights. Cyanobacterial blooms can cause fish kills and are potentially toxic to humans, dogs, water fowl and other animals. They are associated with a reduction in overall biodiversity, and can cause unpleasant surface scums and malodors (Huisman *et al.*, 2018). Cyanobacterial nuisance is a widespread phenomenon in eutrophic urban ponds in The Netherlands (Waajen *et al.*, 2014) and other countries (Mantzouki *et al.*, 2018). Cyanobacterial blooms are expected to increase under climate change (and urbanization) due to an interactive increase in temperature and eutrophication (Moss *et al.*, 2011; Kosten *et al.*, 2012; Paerl & Paul, 2012).

We here studied 83 ponds (reported on in Chapter 5; in collaboration with Jessie M. T. Engelen) and 13 populations of the water flea *Daphnia magna* (Fig. 6, see further) in Flanders. For each pond we assessed the degree of urbanization based on percentage built-up area in the regional (3200 m radius around the pond) and local surrounding of the ponds (50 m radius around the pond). With regard to environmental change associated with urbanization, Engelen (2017, PhD thesis), could not find clear signals of urbanization-associated environmental disturbance gradients (e.g. higher eutrophication or pesticide contamination in urban ponds). While urban ponds showed increased alkalinity and conductivity levels, and reductions in dissolved organic carbon ($mg.l^{-1}$) and total and available nitrogen

($mg.l^{-1}$), significant results disappeared after correcting for multiple-testing (Bonferonni correction). However, the analyses were based on a one-time sampling of all ponds in the region, which possibly reduces the chance of picking up consistent signals. While a one-time sampling strategy is not ideal, we alternatively hypothesize that both urban and rural ponds show extreme variability in environmental conditions, as is generally the case for small ponds (Hassall, 2014; Thornhill *et al.*, 2018a), which might blur clear associations between urbanization and certain environmental axes such as eutrophication, or pesticide and metal pollution.

3.4 *Daphnia*: model organism in ecology and evolutionary biology

We sampled 13 populations of *Daphnia magna* in the region of Flanders (Fig. 5 and Fig. 6), all characterized by a distinct level of urbanization (for detailed information on the sampling locations and percentage built-up area for each pond, see Table 1). We assessed built-up area (BA) at 7 spatial radii around the pond (50-100-200-400-800-1600-3200 m). Based on the regional (3200 m radius) percentages of BA, ponds were classified either being urban (> 10% BA, but see Table 1: all urban ponds had a BA of 12% or more) or rural (< 5% BA; but see Table 1, all rural ponds had a BA of 4.3% or less). Given that BA does not include roads and parking lots, ponds with regional BA levels of 10% or more are already considered very urbanized (shown in Fig. 6b). As in the Speedy design, rural ponds were characterized by at least 20% of Biologically Valuable Area in their regional surroundings. Percentage built-up area at local (100m-400m) correlated well with regional urbanization (3200m; see Fig. S2 of the Supplementary Information of Chapter 3).

Daphnia magna belongs to the family of *Daphniidae* (subphylum: Crustacea, phylum: Arthropoda). While *Daphnia* are distributed worldwide, they are most abundant in temperate regions. *Daphnia* cover a wide variety of freshwater ponds and lakes, but are most commonly found in eutrophic shallow lakes. *Daphnia* has a cyclical parthenogenetic life cycle, characterized by asexual (parthenogenesis) reproduction under favorable conditions and sexual reproduction when conditions deteriorate (photoperiodical changes, extreme high or low temperatures, food limitation, overcrowding; Kleiven *et al.*, 1992). Sexually produced eggs (or 'ephippia'), build up each growing season in the sediment as dormant resting stages that can hatch in subsequent seasons and under good conditions (increased temperature and light availability, low population densities). Both types of reproduction make *Daphnia* extremely useful for ecological and evolutionary studies. The resting stages enable to perform 'resurrection ecology' studies (Angeler, 2007; Frisch *et al.*, 2014; Geerts *et al.*, 2015; Yousey *et al.*, 2018), in which dormant stages from the past are hatched, reared, and scored for certain life-history, morphological, or physiological traits, and compared to current day population trait values. This approach allowed to study for example how *Daphnia* adapted to changed natural and anthropogenic selection pressures such as fish predation (e.g. Cousyn *et al.*, 2001; Pauwels *et al.*, 2007; Stoks *et al.*, 2016), UV-radiation (Oexle *et al.*, 2016), and climate warming (e.g. Geerts *et al.*, 2015;

Yousey *et al.*, 2018). Their asexual reproduction allows to culture them for multiple generations under standard laboratory conditions, generating genetically identical mother-daughter lineages. In this research, I used common garden experiments as an experimental approach. We reared clonal lineages of each pond for multiple generations in the lab, which minimizes maternal effects. Maternal effects can impact responses substantially and bias the observed genetic differentiations among populations. Given their relatively short generation time (ca. 11 days until first reproduction with subsequent juvenile releases every 3 days), high fecundity (under standardized culturing conditions; e .g. 14:10 L:D photoperiod, high food quantity and quality, and medium refreshment every two days), ease of rearing, and parthenogenetic life cycle, *Daphnia* are a very attractive model organism in evolutionary biology..

In response to natural and anthropogenic disturbances, a numerous set of common garden studies showed genetic adaptation in *Daphnia* to natural and anthropogenic stressors or a combination of both (e.g. fish predation: Cousyn *et al.*, 2001; Pauwels *et al.*, 2007; Stoks *et al.*, 2016; cyanobacteria; Hairston *et al.*, 2005; Lemaire *et al.*, 2012; pesticides: Coors *et al.*, 2009; Jansen *et al.*, 2011a, 2015; metals: Barata *et al.*, 2002; Lopes *et al.*, 2006; Ward & Robinson, 2009; Messiaen *et al.*, 2013; temperature; De Meester *et al.*, 2011; Yampolsky *et al.*, 2014; Geerts *et al.*, 2015). Given that *Daphnia* are key mediators of freshwater aquatic ecosystem functions we focus on studying multi-trait adaptation to urbanization in *Daphnia*. Anthropogenic warming, eutrophication, and pollution are prevalent in urban areas and expected to increase under future climate change. Therefore, the results of our study might clarify how *Daphnia* populations persist in city ponds and how this translates into evolutionary adaptations to future climate change, which stressors are mostly driving the observed genetic differentiations, and what the consequences are for freshwater ecological dynamics.

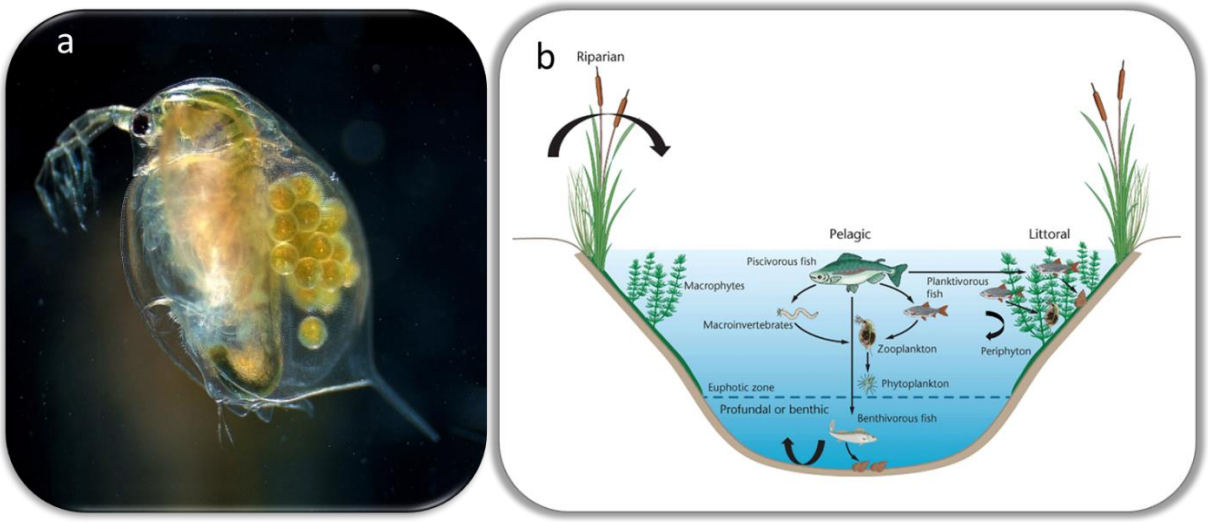

Figure 5. (a) The focal water flea species studied in this project, *Daphnia magna* (photo: Hajime Watanabe). *Daphnia* is a commonly used aquatic species in ecology, evolutionary biology, and ecotoxicology. It has previously been shown that *Daphnia* harbor a high evolutionary responsiveness to natural and anthropogenic environmental changes (e.g. Hairston *et al.*, 2005; Pauwels *et al.*, 2007; Geerts *et al.*, 2015). (b) Given their

importance in the aquatic food web (top-down control of phytoplankton and food source for macro-invertebrates and small fish), *Daphnia* are regarded as crucial interactors in freshwater ponds and pools, contributing to the stabilization of the clear-water phase (Scheffers *et al.*, 1993). Drawing by M. Meerhoff & T. Christensen, from Kosten & Meerhof (2014; bold arrows specify the main nutrient fluxes among lakes zones and with the adjacent terrestrial environment via the riparian zone).

Table 1. *Daphnia magna* populations studied in this PhD. Locations of the *D. magna* study populations including the percentage built-up area (BA) in a radius of 3200 m around each pond, the according urbanization categorization, and the number of experimental lines and clones (i.e. multi-locus genotypes, MLGs) used in the experiment.

Population		**Location**		**Urbanization**	
Pond	Code	Latitude (N)	Longitude (E)	%BA (3200 m)	Cat (3200 m)
Brussel (Kaaienwijk-Dansaert)	*Brxl*	50.855464	4.347334	40.440	urban
Damme (Damme)	*Damm*	51.26207	3.276039	1.126	rural
Gent (Citadelpark)	*GenC*	51.03890	3.723744	24.242	urban
Gent (Muinkpark)	*GenM*	51.04251	3.731611	25.736	urban
Geraardsbergen	*Gera*	50.78420	3.915978	4.382	rural
Kortrijk (Blauwe Poort)	*BppK*	50.81624	3.271563	15.356	urban
Lapscheure (Lapscheure)	*Laps*	51.28253	3.355467	0.637	rural
Leuven (Oude-Meren)	*OudM*	50.86328	4.723935	13.188	urban
Mechelen (Kruidtuin)	*Mech*	51.02402	4.484039	14.125	urban
Meerdaal (Zoete Waters)	*ZwMe*	50.82274	4.653691	2.017	rural
Midden-Limburg (BKN1)	*MidL*	50.98233	5.317858	3.721	rural
Sint-Niklaas (Bufferbekken)	*BuSN*	51.17808	4.161051	12.479	urban
Sint-Niklaas (Fabiola Park)	*FaSN*	51.15623	4.159557	14.995	urban

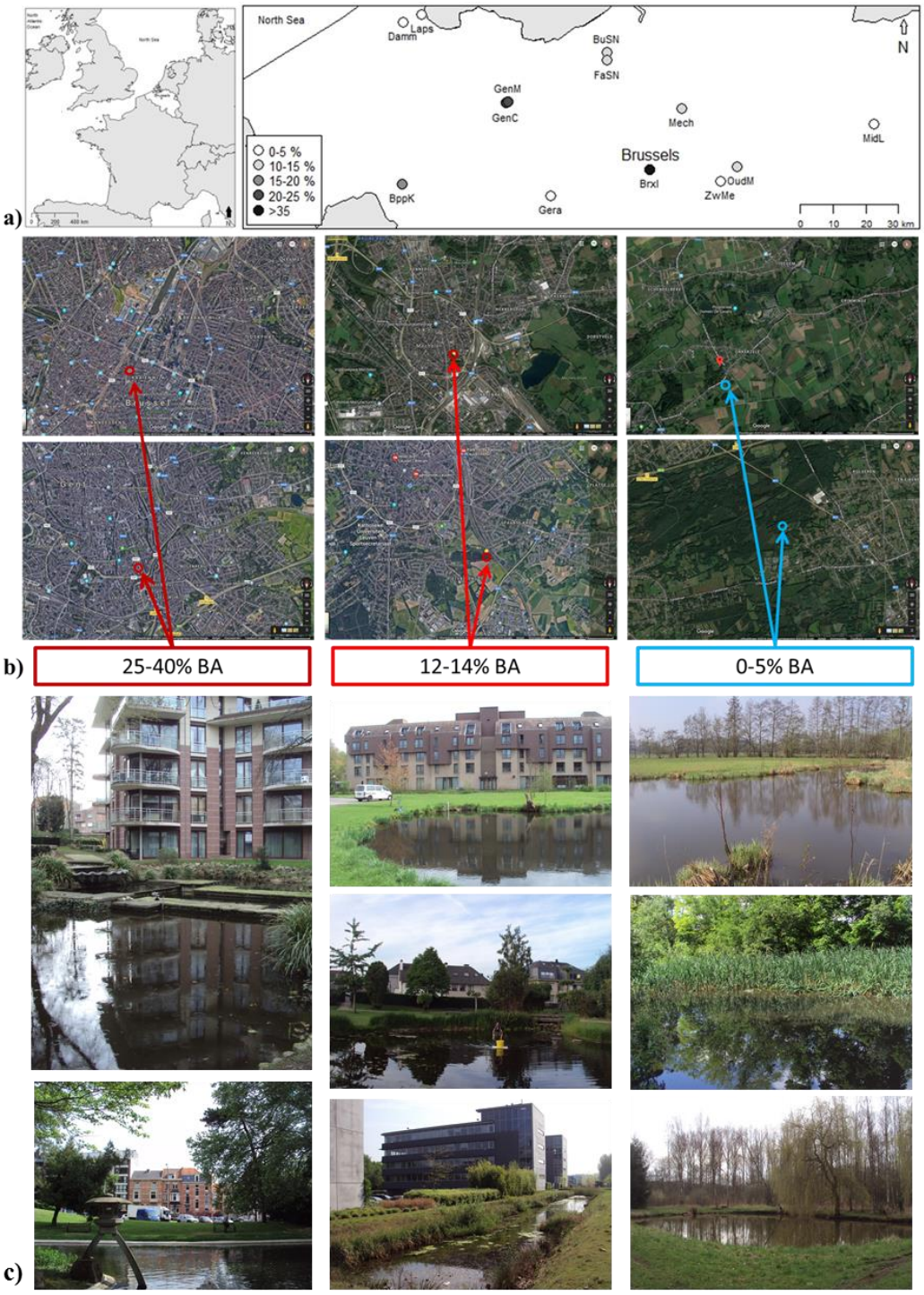

Figure 6. (a) Sampling locations of the 13 *D. magna* populations reported on in this dissertation (indicating the wider region of Europe - inset). (b) Examples of urban and rural ponds for those cases in which urbanization was used as categorical variable (urban > 10% BA, rural < 5% BA) to illustrate ponds characterized by 10% BA and more are already substantially urbanized. Rural ponds were characterized by at least 20% of Biologically Valuable Area in their regional surroundings (3200 m radius) as to assure ponds would be sampled in natural areas, and avoid sampling ponds with low urbanization levels but high agricultural activity. (c) Pictures of a subset of ponds, aligned along the same urbanization categories as in (b).

4 Thesis outline

My research is conceptualized around four hypotheses (Fig. 3):

i) *Urbanization, via the urban heat island effect, impacts the thermal environment of small- to intermediate-sized closed aquatic ecosystems such as freshwater ponds and small lakes.* In specific we aim to test whether urban ponds are significantly warmer than rural ponds, which thermal characteristics are impacted by urbanization (i.e. mean, minimum, maximum temperature, temperature variation, start and end of the growing season) and if so, whether patterns are depending on the season (e.g. winter versus summer), or timing of the day (nighttime versus daytime).

ii) *Compared to rural populations of* Daphnia magna*, urban populations show genetic differentiation in life history and stress physiology, reflecting adaptive evolution to warming*. For example, do urban *Daphnia* genetically differ in their capacity to cope with warming (i.e. do they have a higher Critical Thermal Maximum, or CT_{MAX}, a measure of heat tolerance). Does urbanization result in the evolution of a smaller body size, as expected based on the temperature-size rule? Can we detect shifts in their life-history traits along the slow-to-fast pace-of-life axis? Additionally, we wish to study the relative contributions of evolution, phenotypic plasticity, and the evolution of plasticity to the observed phenotypic trait change In order to assess the latter contributors of phenotypic trait change observed from rural to urban populations, we include a temperature treatment in our common garden experimental designs, reflecting thermal regimes observed in rural and urban ponds in Flanders during summer.

iii) *Urbanization results in zooplankton community trait change that runs in the same direction as observed evolutionary trait change.* In addition, we aim to test what the relative contribution of evolution to the observed community trait change along the studied urbanization gradient is.

iv) *Urban evolution in* Daphnia magna *impacts ecological dynamics, for example zooplankton community trait shifts or predator-prey interactions. Urban evolution, moreover, can lead to cryptic eco-evolutionary dynamics*. More specifically, with regard to predator-prey dynamics we aim to test whether the urban background (and thus evolutionary background) of both predator and prey impact the outcome of their interaction.

Accordingly, this dissertation is structured in three parts: I) **Abiotic conditions** (Chapter 1), II) **Urban evolution** (Chapter 2-4), and III) **Eco-evolutionary dynamics in the city** (Chapter 5-6):

I) Abiotic conditions

In **Chapter 1** I focus on the impact of urbanization and its associated urban heat island effect (Oke, 1973; Aronson *et al.*, 2008; Ward *et al.*, 2015; Wouters *et al.*, 2017) on the thermal characteristics of city ponds in Flanders. This included the assessment of mean, minimum, and maximum temperatures, temperature variation, and the length of estimated growing season.

II) Urban Evolution

In Chapter 2-4 I will elaborate on common garden experiments I performed in the laboratory during which I assessed different stress physiological and life-history traits in a set of *D. magna* genotypes originating from urban and rural ponds. **Chapter 2** includes measurements linked to specific heat adaptation (heat tolerance, body size, and haemoglobin concentration), whereas the next chapters comprise general life-history traits (**Chapter 3**) and stress physiological response variables linked to energy storage compounds and oxidative stress responses (**Chapter 4**). Chapter 3 and 4 both include a more holistic approach in which we explore multivariate genetic differentiation in 'pace-of-life' in urban compared to rural *D. magna* populations (Chapter 3), as well as the presence of overarching pace-of-life syndromes (according to the 'Pace-of-life Syndrome Hypothesis', Ricklefs & Wikelski, 2002; Réale *et al.*, 2010; Sprau *et al.*, 2017) linking suits of life-history and physiological traits, thus focusing on trait covariation patterns (Chapter 4). Additionally, as a step forward in better understanding and elucidating the relative contributions of genetic differentiation and phenotypic plasticity in observed trait shifts, we included comparative analyses on relative contributions of evolution, plasticity, and the evolution of plasticity in Chapters 2 and 3, which was enabled by the experimental set-up including two rearing temperatures (20 and 24 °C) reflecting observed summer temperatures in rural and urban ponds (based on results from Chapter 1, see further).

III) Eco-evolutionary dynamics in the city

Chapter 5 integrates phenotypic data on zooplankton community body size shifts (interspecific trait variation and abundances, based on field measurements, resulting in species sorting dynamics; SPT) in response to increasing urbanization in the surroundings of the pond with intraspecific trait variation data assessed in *D. magna*, including intraspecific trait variation linked to thermal plasticity or other factors (ITV) and genotypic size (genotypic trait variation, GTV; based on laboratory measurements). This chapter aims at testing whether ecological trait shifts (community level responses) parallel evolutionary trait shifts (population level response), and what the relative contribution is of interspecific trait variation (i.e. species turnover, SPT), intraspecific trait variation linked to plasticity to warming or other factors (among population plasticity; ITV), and intraspecific trait variation linked to genetic differentiation (adaptive evolution; GTV) to the observed community trait shift along the urbanization gradient. Finally, in **Chapter 6** we explore how urban evolution impacts important ecological dynamics, such as predator-prey interactions in freshwater ecosystems. We performed a large-scale common

garden rearing experiment, bringing together two focal study species, the water flea *Daphnia magna* and larvae of the damselfly *Ischnura elegans* (a common predator of *D. magna*), and assessed predation rates under simulated heatwave scenarios, as urbanization is expected to coincide with an increased occurrence and intensity of heatwaves (Wouters *et al.*, 2017).

Part 1: Abiotic Environment

Chapter 1

Urban hot-tubs: local urbanization has profound effects on average and extreme temperatures in ponds

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Highlights

- Urbanization impacts closed freshwater ecosystems, causing thermal pollution
- Local urbanization causes increased mean, minimum, and maximum water temperatures
- Estimated growing seasons start earlier and are prolonged in urban ponds
- Effects on temperature parameters are most intense in spring and summer
- The here defined ‘urban hot-tub’ effect should be considered in urban planning

Abstract

While urbanization-driven warming (urban heat island effect, UHI) has been extensively studied and demonstrated for air temperature, UHI effects on water temperature of ponds are unknown. We investigated (1) whether the UHI impacts man-made urban ponds and tested whether urban ponds have higher mean, maximum and minimum water temperatures and lower daily water temperature fluctuations than rural ponds, (2) whether this is related to time of the day (day versus night), season, and urbanization scale (3200 versus 50 m radius around the pond), and (3) whether the approximated length of growing season is prolonged in urban ponds. Temperature loggers were placed in 30 ponds in Northern Belgium, spanning a broad range of urbanization. We found strong evidence of urban-driven warming. Mainly local urbanization (50 m radius) drove temperature differences throughout the year and even more so in spring and summer, with mean summer temperatures being up to 3.04 °C higher in urban compared to rural ponds, and maximum summer temperatures on average up to 3.69 °C higher. Strikingly, daily temperature fluctuated around 2 °C more in locally urban ponds compared to rural ponds in summer. Length of the growing season estimates show prolongation with up to 45 days in locally urban compared to rural systems, mainly due to an earlier start. Generally, our results show that UHIs impact water temperature of ponds. This warming can have profound consequences for biota inhabiting these systems, and should therefore be considered in future urban planning to reduce deterioration of these habitats and improve their socio-ecological value.

INTRODUCTION

More than half of the world's population currently lives in densely concentrated urban cores (UN, 2014). These urban cores generate complex environments, as they integrate a multitude of physical, chemical and climatic gradients (Kaye *et al.*, 2006; Grimm *et al.*, 2008a,b). One consequence of urbanization is a microclimate shift, known as the urban heat island effect (Oke, 1973; UHI, Arnfield, 2003; Ward *et al.*, 2016), which involves an increased ambient temperature in city centers compared with rural areas and the urban fringe. This UHI effect (Bornstein, 1968; Oke, 1973; Voogt & Oke, 2003) is the result of increased land cover by buildings, roads, and other artificial surfaces linked to urbanization and industrialization (Rizwan *et al.*, 2008; Stewart & Oke, 2012). Specific street geometric characteristics, the proportion of artificial surfaces with low albedo values and high warmth emissivity, anthropogenic heat production, and city size influence the strength of the UHI effect and have been used to predict its intensity (Oke, 1982; Voogt & Oke, 2003; Hart & Sailor, 2009; Ward *et al.*, 2016).

While the UHI effect has been extensively studied and shown for air temperatures (Hart & Sailor, 2009; Ward *et al.*, 2016), so far effects on water temperature have been largely overlooked. Recent evidence supports the warming of streams and rivers due to urbanization (Kaushal *et al.*, 2010; Hester & Baumann, 2013). Surrounded by high levels of built-up area and impervious surfaces, rivers and streams in urban areas often receive warm water inflows, especially in summer and during heatwaves, causing thermal pollution (Lieb & Carline, 2000; Herb *et al.*, 2008; Hester & Bauman, 2013). For standing waters such as ponds and shallow lakes, however, no data are available on the UHI effect on water temperature nor on the potential mechanisms behind it. We expect that higher ambient air temperatures in cities, less shading and thus higher solar irradiation will indeed impact the water temperature of ponds in urban areas. Although the high specific heat capacity of water reduces the speed with which temperatures rise in aquatic systems, high thermal inertia also slows down cooling during the night and might thus increase the UHI effect during sufficiently long-lasting warm periods and heatwaves. Because of these slow cooling rates, we expect daily (i.e. measured over 24h periods) temperature fluctuations to be relatively stable in urban ponds during summer, causing a relatively narrow daily temperature range. Larger sized cities (high regional levels of urbanization) can induce a prolonged heat island effect in autumn and winter by trapping heat on a regional scale, thereby creating a warmer microclimate within the city (Schwarz, 2010). Therefore during autumn and winter regional urbanization could induce temperature differences, albeit of a smaller intensity, in ponds situated in large urban areas compared to ponds in large rural areas.

Ponds, pools, and small lakes provide essential habitat for aquatic biodiversity as they often have well-developed aquatic vegetation supporting multiple organism groups such as amphibians and insects (Williams *et al.*, 2004; Hassall, 2014; Hill *et al.*, 2017). In urbanized areas, ponds are often created by humans for their esthetic and recreational value (e.g. garden ponds, city park ponds) or with

a functional purpose (e.g. storm water wet ponds which capture storm water runoff from impervious surfaces to mitigate flooding and improve water quality in urban areas), while the biodiversity value of these systems is secondary. However, recent studies emphasize the importance of urban ponds for aquatic biodiversity and the fact that biodiversity should be taken into account in pond design (e.g. Hassall, 2014; Hill *et al.*, 2017; Gianuca *et al.*, 2018). Temperature is one of the most important drivers of physiological processes and phenology in nature (Angilletta, 2009), and higher water temperatures due to the UHI effect might therefore have a strong impact on the aquatic biota (Brans *et al.*, 2017b). The early onset and extended duration of the growing season in urban areas (Zhang *et al.*, 2004; Zipper *et al.*, 2016) triggers early flowering in plants and causes phenological shifts in many ectotherms (Neil & Wu, 2006; Kaiser *et al.*, 2016). In aquatic systems, the impact of the UHI effect will depend on its effects on average and maximum temperatures (e.g. during heatwaves), seasonality, and amplitudes of temperature fluctuations. As a consequence of these changes, the length of the growing season for aquatic organisms might be profoundly impacted by the UHI, as already observed in streams (Nelson & Palmer, 2007).

In this study, we tested whether the UHI also affects the water temperature of urban ponds. We monitored water temperature in 30 ponds situated over a broad range of urbanization using temperature loggers during a 12 month period. We hypothesized (1) that urban ponds exhibit significantly higher temperatures than rural ponds, (2) that the difference in mean, maximum and minimum temperature between urban and rural systems strongly depends on the scale at which urbanization is assessed (local versus regional), the time of day (day versus night), and the season, (3) that daily variation in temperatures is more buffered in ponds in larger urban areas (regional scale) compared to rural ponds, and (4) that the approximated growing season starts earlier and ends later in urban compared to rural ponds.

MATERIALS AND METHODS

Study design and data acquisition

Study area, urbanization levels, and temperature monitoring

We installed 30 temperature loggers (HOBO TidbiT v2 Water Temperature Logger UTBI-001) in 30 ponds along a well-defined urbanization gradient (Fig. 1; Table S1 in Supplementary Information). All selected ponds were shallow (< 3 m maximum depth), with a focus on small to intermediate sized (all studied ponds < 2 ha, according to Hassall *et al.*, 2014; all ponds except two < 0.5 ha) man-made city ponds, park ponds, garden ponds and ponds in (semi-)natural areas (e.g. ponds created as part of nature conservation activities or old farmland ponds), not connected to other water-related infrastructure. Percentage built-up area (BA) was derived from the Large Reference Database (LRD; available via the Flemish Institute for Geographic Information, FIGA) and was used as a proxy for the level of

urbanization (Table SI1, see also Brans *et al.*, 2017a; Gianuca *et al.*, 2018). LRD is an object-oriented, vector-based map with scale of 1/250. The accuracy of the location of buildings varies between 5 cm (when measured *in situ*) and 20 cm (when based on aerial pictures). This metric excludes roads and parking lots. Therefore, a BA of 10% already indicates a high amount of urbanization. Ponds were categorized according to percentage BA around the pond into being urban (> 10% BA), semi-urban (5 - 10% BA), and rural (< 5% BA) ponds at both a local (50 m radius around the pond, excluding the pond itself) and regional (3200 m radius) scale (in line with Piano *et al.*, 2017). This entails that rural ponds at the local scale are purely defined based on percentage BA within a 50 m radius, so that this category groups ponds in nature reserves, farmland ponds, park and garden ponds in cities provided that locally the percentage of built-up area is low. The number of ponds in the local and regional urbanization categories was balanced (Table S1). Temperature loggers were attached to a floating device in order to keep the loggers at a depth of 15 cm below water surface (construction information see Fig. S1 in Supplementary Information). Temperature was logged at 15 minute intervals (96 measurements/day) for a period of one year (November 2014 - November 2015, 367 days). Data output from each logger was obtained using the HOBO digital software (HOBOware, ONSET). Example temperature profiles for the region of Ghent and Leuven are given in Figure 2.

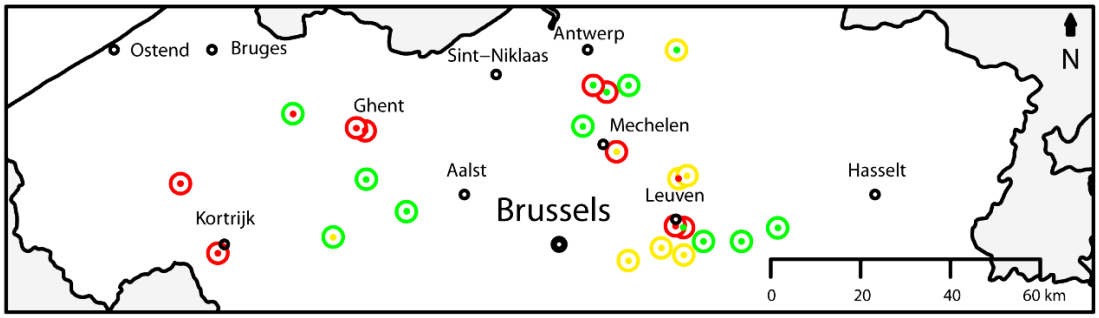

Figure 1. Locations of monitored ponds (n = 30), depicting the region of Flanders (Belgium, with the capital city Brussels). Urbanization levels (urban: red, semi-urban: yellow, rural: green) are given for the near (50 m radius, inner point) and regional (3200 m, outer circle) surroundings of the ponds.

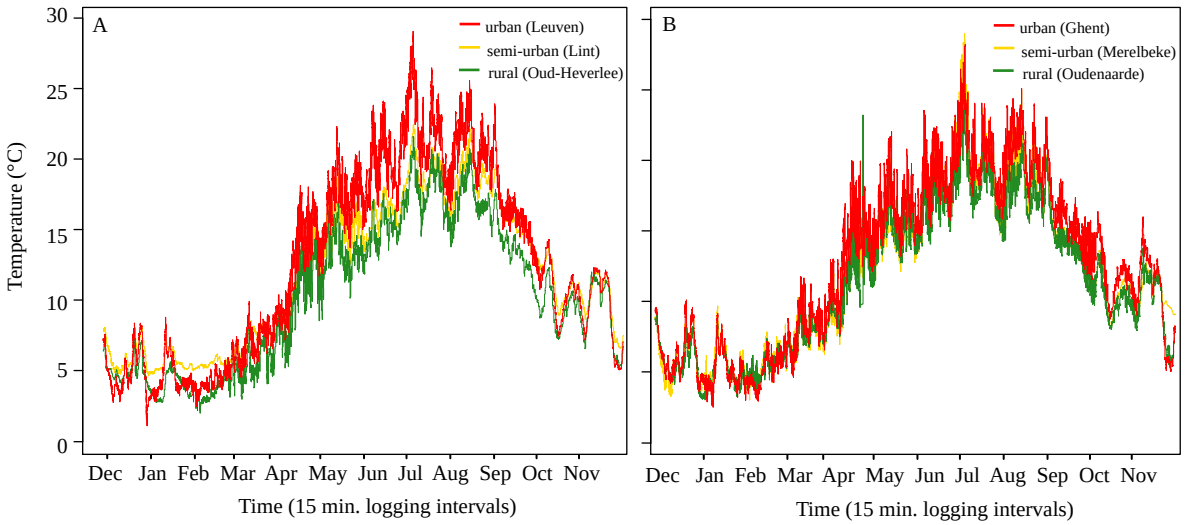

Figure 2. Temperature profiles (°C, measured at 15 min. intervals; period November 2014- November 2015) for an urban (red), semi-urban (yellow), and rural (green) pond in the regions of (A) Leuven, and (B) Ghent.

Temperature response variables

Based on the raw data from the temperature loggers we calculated eight temperature regime proxies for all ponds: mean, minimum and maximum daily temperature, daily temperature range, daytime mean temperature, daytime temperature range, nighttime mean temperature, and nighttime temperature range. Daily refers to the 24h cycle, thus combining daytime and nighttime observations. Daily temperature range (DTR) was calculated as the difference in minimum and maximum daily temperatures. Time intervals for calculating daytime and nighttime temperature means and ranges were based on average sunset and sunrise times for each month (see Table S2). Additionally, three parameters related to growing season were calculated: start, end, and length of the growing season. Length of the growing season (LGS) was approximated as the number of days the mean daily water temperature exceeded a certain threshold value (8, 10, 12, 14, 16, 18, or 20 °C); these temperatures span a range corresponding to ecologically relevant thermal stimuli for organisms known from literature (Jobling, 1981; Ward & Stanford, 1982; Schwartz & Hebert, 1987) . Start of the growing season was the first of three consecutive days at which water reached a temperature higher than the respective threshold value. End of the growing season was considered as the first day at which water temperature no longer exceeded the threshold value.

Data analysis

From the initial set of 30 temperature loggers, six loggers were removed by a third party and another six loggers were removed from analyses due to logging errors (accidental measuring of air or sediment temperatures or improper functioning of the device). Raw data of the remaining 18 temperature loggers were checked for inconsistencies. Three ponds showed obvious errors for longer time periods (perhaps because they were temporarily exposed to the air or because of software problems); these time periods

were treated as missing values (Mechelen: n = 129 days, Rotselaar 1: n = 90 days, Rotselaar 2: n = 212 days). Mean daily temperature values that exceeded 3 standard deviations from mean annual temperatures were excluded because such extreme values are indicative of temperature measurements of loggers exposed to the air (DeWeber & Wagner, 2014). This resulted in the removal of 1 to 14 daily temperature observations in a total of 16 ponds.

To test for an effect of local (50 m radius) and regional (3200 m radius) levels of urbanization on the different temperature response variables, a linear mixed-effect model was carried out for each parameter separately, using the 'lme4' package (Bates *et al.*, 2015; R Development Core Team, 2016). To test for seasonal changes in the effects of urbanization on temperature, we included season as main effect and season × 50 m urbanization and season × 3200 m urbanization as interaction effects. Seasons were delineated by their calendar-based dates (winter: 21 December 2014 – 20 March 2015; spring: 21 March 2015 – 20 June 2015; summer: 21 June 2015 – 20 September 2015; autumn: 21 September 2015 – 20 December 2015). Pond identity and day of the year were included as random effects in all analyses. We corrected for the confounding effects of depth and area of the ponds by including them as covariates in the models. In order to estimate the effect of different levels of urbanization within seasons, Tukey post-hoc comparisons were produced using the 'lsmeans' package (Lenth, 2016; R Development Core Team, 2016). All statistical analyses were carried out using R software version 3.3.2 (R Core Team, 2016). P = 0.05 was used as threshold for significance.

RESULTS

Temperature means, minima and maxima

We found a significant main effect for urbanization at the local scale on mean daily water temperatures (linear mixed-effect model T_{mean}: $p < 0.001$, Table 1). Post-hoc comparisons revealed that mean daily temperatures were significantly higher in urban and semi-urban ponds compared to rural ponds ($\Delta T_{mean, semi\text{-}urban - rural} = +1.41$ °C, $p = 0.025$; $\Delta T_{mean, urban - rural} = +1.74$ °C, $p = 0.014$; 15-min. interval temperature profiles for an urban, semi-urban, and rural pond located in the regions of Leuven and Ghent are depicted as an example in Fig. 2A and B, respectively). Additionally, there was a highly significant season-dependent effect of urbanization on all tested temperature parameters at both the local (50 m) and regional (3200 m) scale (Table 1), however, post-hoc comparisons revealed no significant differences between regional urbanization classes within seasons. Post-hoc comparisons of local urbanization within seasons showed that mean daily temperatures in spring and summer differed significantly between locally rural and urban ponds ($\Delta T_{mean, urban - rural} = +2.81$ °C in spring, $p < 0.001$; $\Delta T_{mean, urban - rural} = +3.04$ °C in summer, $p < 0.001$; Fig. 3A), and between rural and semi-urban ponds ($\Delta T_{mean, semi\text{-}urban - rural} = +1.82$ °C in spring, $p = 0.006$; $\Delta T_{mean, semi\text{-}urban - rural} = +2.21$ °C in summer, $p = 0.001$). We found similar patterns using daytime and nighttime mean temperatures as explanatory

variables in the model, although temperature differences were less pronounced for both compared to daily temperatures (Supplementary information, Table S3 and Table S4).

Overall, models on daily minima and maxima showed similar results (Table 1, Fig. 3B,C). T_{max} was significantly higher throughout the year in locally urban ponds compared to locally rural ponds ($\Delta T_{max,\ urban-rural}$ = +2.23 °C, p = 0.009). Here too, temperature differences were more pronounced in spring and summer: daily maxima in locally semi-urban and urban ponds were 1.89 °C up to 2.39 °C warmer in spring (p = 0.014 and p < 0.001), and 3.28 °C up to 3.69 °C warmer in summer (p = 0.003 and p < 0.001) compared to rural ponds, respectively (Fig. 3B). Daily minima were significantly higher for locally semi-urban and urban compared to rural ponds ($\Delta T_{min,\ semi-urban-rural}$ = +1.28 °C, p = 0.011; $\Delta T_{min,\ urban-rural}$ = +1.11 °C, p = 0.04). Likewise, the interaction effect with season showed the intensity of this effect was highest in spring and summer (Table 1, Fig. 3C): semi-urban and urban ponds showed higher daily minima compared to rural ponds at the local scale in spring ($\Delta T_{min,\ semi-urban-rural}$ = +1.74 °C, p = 0.001; and $\Delta T_{min,\ urban-rural}$ = +2.25 °C, p < 0.001) and summer ($\Delta T_{min,\ semi-urban-rural}$ = +1.91 °C, p < 0.001; and $\Delta T_{min,\ urban-rural}$ = +2.05 °C, p < 0.001; Fig. 3C).

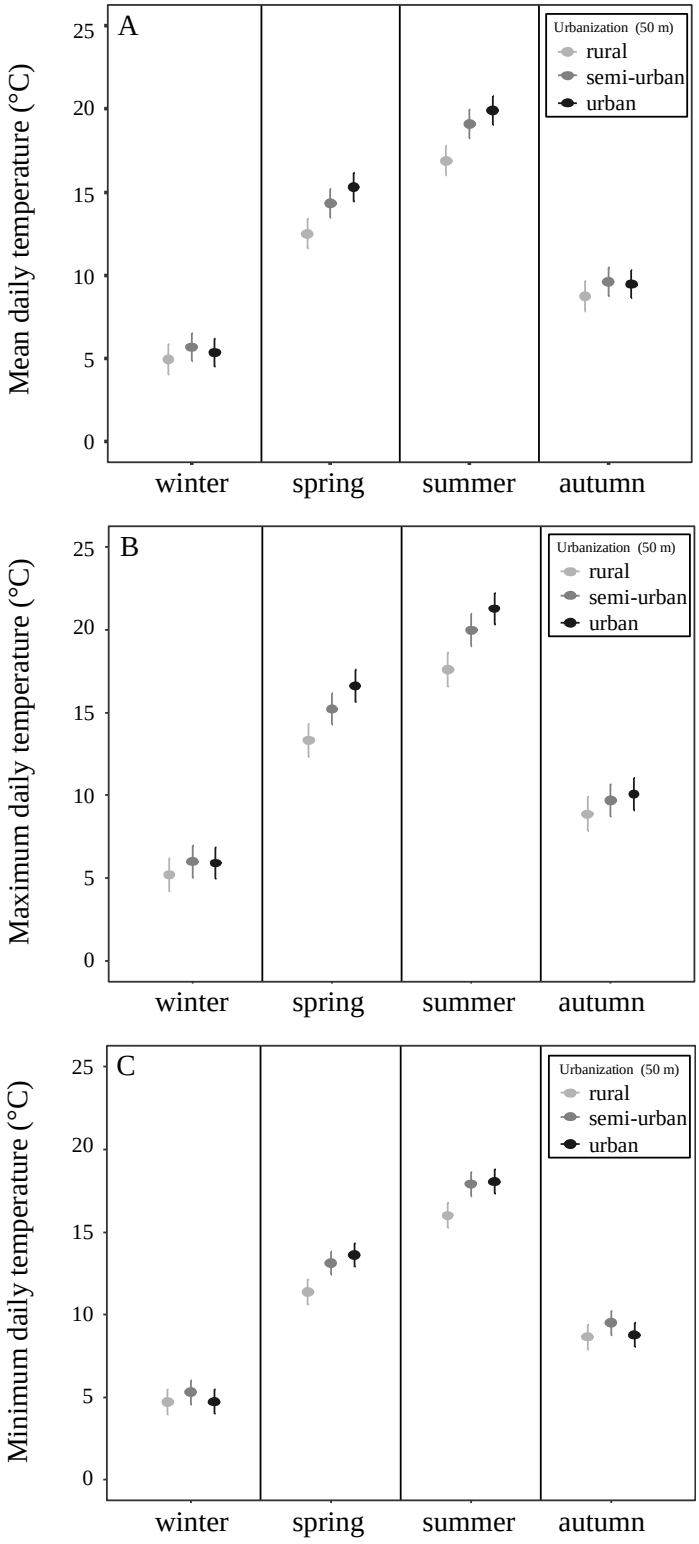

Figure 3. Mean (a), maximum (b), and minimum (c) daily temperature (°C) ± 2 SE measured in rural (light grey), semi-urban (grey), and urban (black) ponds. Urbanization level (measured as % built-up area) was assessed at a radius of 50 m around each pond.

Temperature range

We found a highly significant season-dependent effect of urbanization on daily (DTR) and nighttime (NTR) temperature ranges at both local (50 m) and regional (3200 m, Fig. 4) urbanization scales, as well as a significant main effect of local urbanization (Table 1, DTR; Table S4, NTR, Supplementary Information). Locally urban ponds showed on average a 1.29 °C higher DTR compared to rural ponds ($p = 0.019$, Fig. 4A). Furthermore, the interaction of local urbanization within seasons (Table 1) revealed that locally urban ponds had a significantly higher DTR in summer (+1.95 °C, $p = 0.001$), in spring (+1.14 °C, $p = 0.037$), and in autumn (+1.28 °C, $p = 0.021$) compared to rural ponds (Fig. 4A). Urban ponds showed also a significantly higher DTR compared to semi-urban ponds in autumn (+1.29 °C, $p = 0.018$) and summer (+1.42 °C, $p = 0.010$) (Fig. 4A). Regionally urban ponds had a significantly lower DTR (-1.44 °C, $p = 0.0071$) in summer compared to semi-urban ponds (Fig. 4C). The results for daytime temperature ranges were similar as those obtained for daily temperature ranges (Table S3 in Supplementary Information).

Nocturnal temperature range (NTR), differed slightly from DTR in its response to urbanization (results given in Table S4, Supplementary Information). Overall, temperature range differences were less pronounced during the night than during the day (Fig. 4). Locally urban ponds showed a 0.70 and 0.75 °C larger NTR compared to semi-urban ($p = 0.015$) and rural ponds ($p = 0.009$). Furthermore, a significantly higher NTR was observed in autumn, spring and summer for locally urban compared to locally semi-urban (+0.90 °C, $p = 0.003$; +0.62 °C, $p = 0.031$; and +0.90 °C, $p = 0.003$; respectively, Fig. 4B,) and locally rural ponds (+0.91°C, $p = 0.003$; +0.64 °C, $p = 0.029$; and +1.05 °C, $p = 0.001$; respectively, Fig. 4B). Regional urbanization revealed the opposite pattern. Compared to semi-urban ponds, regionally urban ponds had a 0.59 °C and 1.06 °C lower NTR in autumn ($p = 0.033$, Fig. 4D) and summer ($p < 0.001$; Fig. 4D), respectively, and a 0.58 °C lower NTR compared to rural ponds in summer ($p = 0.042$, Fig. 4D).

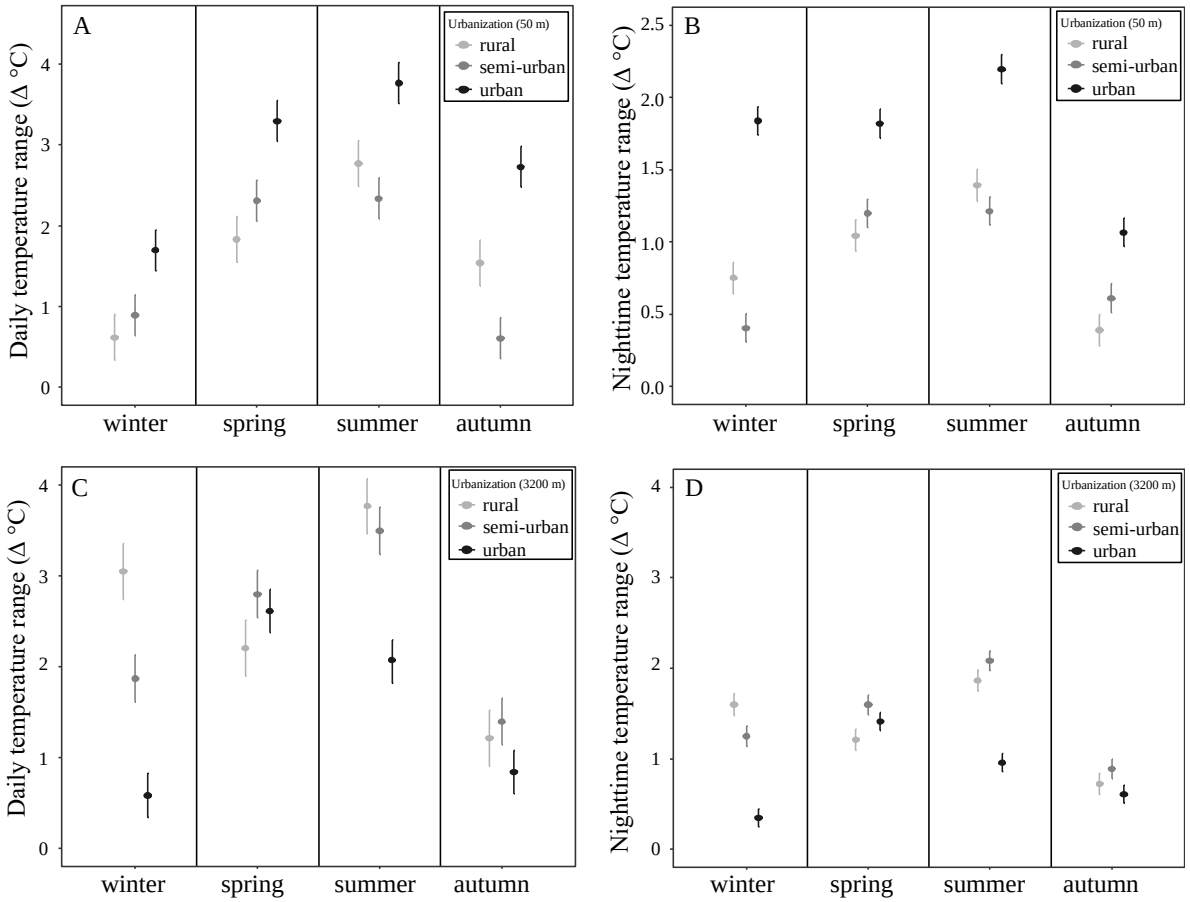

Figure 4. Mean daily temperature range (Δ °C; A,C; DTR) ± 1 SE and nocturnal temperature range (Δ °C; B,D; NTR) ± 1 SE measured in rural (light grey), semi-urban (grey), and urban (black) ponds. Urbanization levels were assessed at a radius of 50 m (A,B) and 3200 m (C,D) around the pond.

Start, end and length of estimated growing season

Start of the growing season (Julian day) was highly affected by local urbanization (linear mixed-effect model: $p < 0.001$, Table 2, Fig. 5). This effect also depended on the specific threshold temperature used to define the start of the growing season (threshold temperature × local urbanization, $p = 0.019$, Table 2). On average, the growing season in locally rural ponds started approximately 24 and 16 days later than in urban ($p = 0.003$) and semi-urban ponds ($p = 0.023$), respectively. Post-hoc comparisons between local urbanization levels at different threshold temperatures revealed a significantly delayed start for rural ponds compared to semi-urban ponds at threshold temperatures of 8 °C ($p = 0.040$), 14 °C ($p = 0.043$), 16 °C ($p = 0.010$), and 18 °C ($p = 0.004$) (Fig. 5), and for rural versus urban ponds from 8 °C ($p = 0.008$) and 14°C ($p < 0.001$) up to 20 °C ($p < 0.001$, $p < 0.001$, $p = 0.031$ for all remaining temperatures; Fig. 5).

The end of the growing season (Julian day) was significantly affected by regional urbanization rather than by local urbanization, but this depended on the temperature threshold considered (threshold temperature × regional urbanization, $p = 0.004$, Table 2). Post-hoc comparisons between regional

urbanization categories at different temperature thresholds revealed no significant differences between urban, semi-urban or rural ponds, however.

Length of the growing season was affected by local urbanization (linear mixed-effect model: $p = 0.035$, Table 2, Fig. 6), and there were interaction effects of both local and regional urbanization with threshold temperature ($p = 0.024$ and $p = 0.008$, respectively; Table 2). Locally rural ponds tended to have a shorter growing season (LGS = 123 days) compared to urban ponds (LGS = 168 days; post-hoc test, marginally non-significant: $p = 0.07$). The growing season was significantly longer for locally urban ponds compared to locally rural ponds for threshold temperatures of 14 °C (+64 days, $p = 0.015$), 18 °C (+62 days, $p = 0.019$) and 20 °C (+59 days, $p = 0.038$) (Fig.6). Yet, no significant differences in length of the growing season were found among regional urbanization categories at any of the temperature threshold values after performing post-hoc tests.

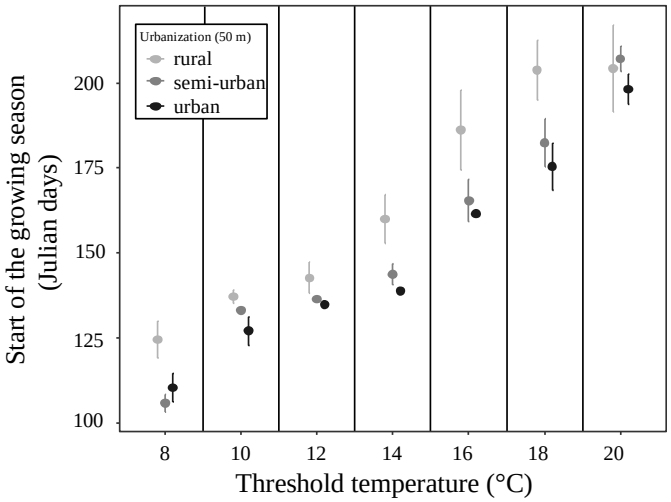

Figure 5. Mean start of the estimated growing season (Julian day) ± 1 SE for rural (light grey), semi-urban (grey), and urban (black) ponds. Urbanization levels were assessed at a radius of 50 m around the pond. Day number is Julian day at which water temperature exceeded the threshold temperature. Threshold temperatures ranged from 8 °C to 20 °C.

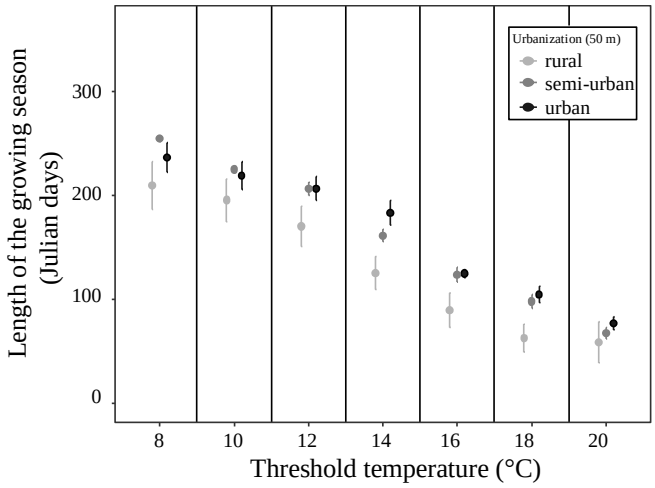

Figure 6. Mean length of the estimated growing season (days) ± 1 SE for rural (light grey), semi-urban (grey), and urban (black) ponds. Urbanization levels were assessed at a radius of 50 m around the pond. Threshold temperatures ranged from 8 °C to 20 °C.

Tables

Table 1. Results of the mixed-effect models testing the effect of urbanization, season, and all two-way interactions on mean, minimum, and maximum daily (24h) temperatures (°C), and daily temperature range (Δ °C, DTR). Depth and area of the ponds were included as covariates in all models. Significant results ($p < 0.05$) are given in bold.

	Daily temperature (°C)									**DTR (Δ °C)**		
	Mean			***Minimum***			***Maximum***					
Fixed effects	**F**	**Df**	**p**	**F**	**Df**	**p**	**F**	**Df**	**p**	**F**	**Df**	**p**
Urbanization level 50 m	**18.017**	**2**	**< 0.001**	**18.238**	**2**	**< 0.001**	**17.933**	**2**	**< 0.001**	**11.980**	**2**	**0.002**
Urbanization level 3200 m	0.005	2	0.997	0.701	2	0.704	0.266	2	0.876	5.675	2	0.059
Season	**1269.865**	**3**	**< 0.001**	**1206.495**	**3**	**< 0.001**	**1260.701**	**3**	**< 0.001**	**623.014**	**3**	**< 0.001**
Depth	2.185	1	0.139	1.353	1	0.245	2.981	1	0.084	0.945	1	0.331
Area	1.598	1	0.206	**4.070**	**1**	**0.044**	0.879	1	0.348	0.718	1	0.397
Urbanization level 50 m x season	**1012.378**	**6**	**< 0.001**	**937.751**	**6**	**< 0.001**	**780.198**	**6**	**< 0.001**	**202.873**	**6**	**< 0.001**
Urbanization level 3200 m x season	**167.878**	**6**	**< 0.001**	**201.912**	**6**	**< 0.001**	**195.280**	**6**	**< 0.001**	**466.525**	**6**	**< 0.001**

Table 2. Results of the mixed-effect models testing the effect of urbanization, threshold temperature, and all two-way interactions on start, end, and length of the growing season. Depth and area of the ponds were included as covariates in all models. Significant results ($p < 0.05$) are given in bold.

	Length of growing season (days)			**Start of growing season (Julian day)**			**End of growing season (Julian day)**		
Fixed effects	χ^2	**Df**	**p**	χ^2	**Df**	**p**	χ^2	**Df**	**p**
Urbanization level 50 m	**6.678**	**2**	**0.035**	**20.443**	**2**	**< 0.001**	2.467	**2**	0.291
Urbanization level 3200 m	0.829	2	0.660	0.3729	2	0.830	1.372	2	0.504
Threshold temperature	**1733.628**	**3**	**< 0.001**	**1130.139**	**3**	**< 0.001**	**625.942**	**2**	**< 0.001**
Depth	2.472	1	0.116	1.210	1	0.271	2.668	1	0.102
Area	0.229	1	0.632	0.313	1	0.576	0.166	1	0.683
Urbanization level 50 m x threshold temperature	**23.405**	**6**	**0.024**	**24.137**	**6**	**0.019**	14.277	**6**	0.283
Urbanization level 3200 m x threshold temperature	**26.678**	**6**	**0.008**	4.777	6	0.965	**28.869**	**6**	**0.004**

DISCUSSION

We found strong evidence of urbanization-driven warming in man-made urban ponds in Flanders, confirming our hypothesis that urban heat islands can cause thermal pollution in intermediate sized ponds in cities. In general, especially local urbanization (i.e. within a radius of 50 m around the pond) was shown to be important for aquatic urban heat islands (UHIs). Local urbanization influenced all temperature parameters considered in this study and mainly affected pond temperatures in spring and summer. These results are comparable with what has been reported for terrestrial UHIs measured on air temperature (Arnfield, 2003; Ward *et al.*, 2016). Regional urbanization levels (i.e. at a radius of 3200 m), however, had no significant effect on mean temperatures and only affected daily and nocturnal temperature ranges in our study ponds. The smaller air temperature differences during autumn and winter likely do not lead to a substantial temperature difference in aquatic systems. Our results, however, are in strong support of anthropogenic warming of freshwater habitats, which should be considered in future urban studies.

The effects of local urban warming on pond temperature parameters were profound and most intense in spring and summer. Mean temperatures in urban ponds were up to 3.04 °C higher than in rural ponds during spring and summer. During these seasons average temperature maxima differed up to 3.69 °C between locally urban and rural ponds. Minimum summer temperatures were higher in locally semi-urban and urban ponds when compared with rural ponds, (temperature elevations ranging between 1.74 °C and 2.25 °C). These results are in line with what is expected based on the occurrence of urban heat islands and the presence of extreme temperature events in cities, which are well-documented in terrestrial systems, also for the study region (Christidis *et al.*, 2015; De Ridder *et al.*, 2015; Ward *et al.*, 2016). De Ridder *et al.* (2015) reported summer mean day air temperature differences between rural and urban sites ranging from +1.5 °C up to +4.5 °C during summer, depending on city size and population density (with extreme differences up to +8 °C). In our dataset, local rural ponds also included city ponds in green areas (typically park ponds and garden ponds). One well-known phenomenon that might contribute to the fact that the impact of the degree of urbanization on pond temperatures mainly acts at the local levels is the so-called park cooling effect in cities. Green areas in the city, such as parks and vegetated patches, induce cooling due to evapotranspiration of trees and larger bushes, reducing temperatures in their immediate surroundings (Dimoudi & Nikolopoulou, 2003; Yu & Hien, 2006; Önder & Akay, 2014; Alavipanah *et al.*, 2015) . Ponds situated in these small plots of urban greenery might benefit from this cooling effect. This causes temperature differences between ponds in local urban versus locally more natural (e.g. parks) to increase. As temperatures and foliage cover decrease during autumn and winter, the overall UHI intensity, together with the park cooling effect, declines. These two factors (i.e. seasonal temperature decrease and disappearance of vegetation) potentially explain the seasonality of our results and the absence of an effect of local urbanization on the aquatic UHI in autumn or winter. Because of this strong dependence on urbanization scale for the

aquatic UHI, and the fact that the immediate surroundings (a 50 m buffer around the pond) impacts pond water temperature more and in a different way than regional urbanization, we suggest to use the term "urban hot-tub effect" for this urbanization-driven warming effect of urban ponds.

Strikingly, temperature fluctuated up to 2 °C more over the course of a day (24h period) in locally urban compared to rural ponds in summer. Although we did not investigate the mechanisms behind the UHI effect on water temperature of urban ponds, the larger daily fluctuations in locally urban ponds compared to rural ponds could be due to a combination of high peak flow temperatures caused by runoff, higher air temperatures, and higher solar irradiation during the day, while locally rural ponds may often be buffered from extreme temperatures thanks to the shading effect and evapotranspiration that trees around the ponds provide during summer (Yu & Hien, 2006; Alavipanah *et al.*, 2015). While we have no data on shading for the ponds presented in the current study, in another study comprising environmental data of 81 ponds sampled over the same gradient in urbanization, we did find a significant negative correlation between urbanization and percentage shade, which might give an indication that urban ponds are less shaded, thereby driving the thermal pollution (Gianuca *et al.*, 2018). In contrast, ponds in regionally urbanized areas showed less temperature fluctuation (up to 1.44 °C in summer) compared to semi-urban ponds during daytime, and compared to semi-urban and rural ponds during nighttime, which can potentially be explained by the city-size effect (Oke, 1973). Large amounts of built-up area at a regional scale may buffer air temperature fluctuations, compared to more open semi-urban and rural areas (suburban garden ponds, farmland ponds, nature reserves), due to the substantial amount of heat trapped during the day and the slower cooling rate at night (as a result of daytime heat absorption and nighttime heat release by buildings).

We found a profound earlier start of the estimated growing season in urban systems (up to 24 days difference) for threshold temperatures ranging between 12 and 20 °C. The length of the growing season was on average prolonged with 45 days in urban systems, mainly due to its earlier start. When setting a temperature threshold of 14 °C, which is a general temperature for the start of growth in many small aquatic invertebrates such as cladocerans (Kryuchkova, 1973; Metzke & Pederson, 2012), this effect even increased to 64 days. The observed increased temperatures in urban ponds in combination with the estimated prolonged length and earlier start of the growing season has important implications for organisms' physical conditions, and can potentially disturb life history and phenology of freshwater species, similarly to reports in terrestrial systems (Neil & Wu, 2006; Kearney *et al.*, 2010). Alternatively, for those organisms for which these changes do not impose stress, an increased length of the growing season may potentially boost production and fitness.

The hottest temperature observed in our study was 34.2 °C. This is an extremely stressful temperature for many aquatic invertebrates (Dallas & Rivers-Moore, 2012; Houghton & Shoup, 2014). Temperature extremes lead to slower development and growth rates (Hester & Bauman, 2013; Somers

et al., 2013). As a result, a pronounced increase in temperature often leads to a decrease in taxon richness and a loss of taxa sensitive to high temperatures and low oxygen levels (Beitinger *et al.*, 2000; Nelson & Palmer, 2007; Jones & Hunt, 2010) at the community level. In a parallel study focusing on the same urbanization gradient in Flanders, Piano *et al.* (2017) observed a reduction in terrestrial carabid beetle species with low thermal optima in locally urbanized sites compared to locally rural sites. In addition to direct impacts, it is likely that a temperature-mediated dominance of cyanobacterial blooms, which are known to be toxic for zooplankton, amphibians, and vertebrates (Paerl *et al.*, 2001; Jöhnk *et al.*, 2008; Paerl & Huisman, 2009), causes a further deterioration of biological diversity and functioning in aquatic ecosystems. The higher temperatures observed by us in urban compared to rural ponds are potentially harmful to organisms, and might cause important functional shifts in communities of organisms inhabiting urban ponds (Lieb & Carline, 2000; Brans *et al.*, 2017a).

Both with respect to the change in mean and maximum temperature as well as in length of the growing season, the effect of local urbanization-driven microclimate warming almost equals the equivalent of 100 years of climate change under IPCC scenario RCP8.5 (IPCC, 2014, Meyer *et al.*, 2014). As has been suggested earlier (Grimm *et al.*, 2008a,b; Youngsteadt *et al.*, 2015), it may therefore be relevant to use urbanization gradients as a space-for-time proxy for climate change studies. Our observations also suggest that migration of populations to track climate change may in practice not only involve movements from lower to higher latitudes or altitudes but perhaps also dispersal from urban populations to rural sites. Species that can adapt to cities would then have an advantage in their response to climate change. Finally, also in the absence of climate change, the temperature-induced changes in phenology of urban versus rural populations may have important ecological consequences, as urban populations may develop earlier in the season and may then through dispersal influence rural populations.

Typically in urban design and planning, ponds are considered to help minimize the effects of UHIs by reducing increased air temperatures (Völker *et al.*, 2013; Gunawardena & Kershaw, 2016). It is often overlooked that these pond systems themselves are also influenced by UHIs. Pond managers might efficiently reduce aquatic urban heat island effects by increasing green, vegetated, areas around the ponds. Like urban green infrastructure (Chiesura, 2004; Tzoulas *et al.*, 2007), ponds and pools are an important source of urban biodiversity (Bolund & Hunhammar, 1999; Hill *et al.*, 2017)), and directly and indirectly contribute to human wellbeing (Chiesura, 2004; Matsuoka & Kaplan, 2008). Proper planning and management of ponds (e.g. inclusion of vegetated, fish-free, zones; non-artificial substrates; reduction of bank-angle steepness; increased depth; vegetated areas in the close proximity of the pond border: Hassall, 2014; Gianuca *et al.*, 2018), in urban landscapes may stimulate these ecosystem services and may prevent that pond habitats deteriorate because they are subject to extreme temperature conditions.

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Supplementary Information "Urban hot-tubs: Local urbanization has profound effects on average and extreme temperatures in ponds"

Figures

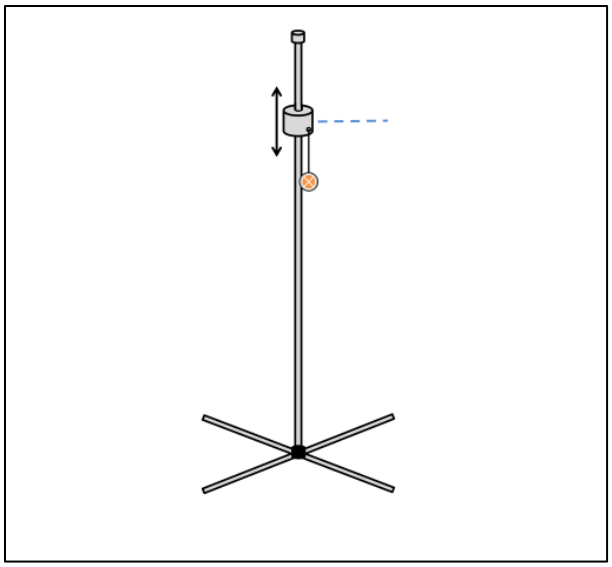

Figure S1. Graphical representation of the construction containing the floating device to which the logger (orange) was attached (15 cm depth from water level, marked by the dashed lines).

Tables

Table S1. Sampling locations (ordered according to regional location), depth and area of the ponds (n = 24, 6 loggers were removed by a third party). Percentage built-up area (BA) at 50 m and 3200 m around the ponds is given, reflecting the local and regional urbanization level respectively (U = urban, SU = semi-urban, R = rural/natural). Regionally rural ponds need to meet the additional criterion to consist of at least 20% of Biologically Valuable Area (BVA, assessed at 3200 m). In case this requirement is not fulfilled, ponds are categorized semi-urban, given that they occur in agricultural areas.

Pond	Province	Depth (m)	Area (m^2)	Volume ($L.m^{-2)}$	Longitude	Latitude	%BA 50 m	Urbaniz. local	%BA 3200 m	% BVA 3200m	Urbaniz. regional
Edegem	Antwerp	1.8	4800	9000	51.148488	4.437716	7.468	SU	13.907	12.536	U
Heindonk	Antwerp	1	275	275	51.06647	4.40517	8.085	SU	5.240	38.526	SU
Lier	Antwerp	1.5	450	675	51.148271	4.550157	0	SU	8.546	16.650	SU
Lint	Antwerp	1	600	600	51.134725	4.480525	5.033	SU	11.273	13.888	U
Linter	Antwerp	0.8	100	80	50.862833	5.022519	0	SU	2.323	18.552	SU
Mechelsbroek	Antwerp	0.7	60	42	51.015522	4.512072	0	R	11.843	24.536	U
Vorselaar	Antwerp	1.4	80	112	51.21917222	4.702294444	9.293	SU	3.574	33.550	R
Aalter	East Flanders	1	900	900	51.091274	3.487203	14.905	U	4.125	13.880	SU
Bourgoyen	East Flanders	0.8	4	3.2	51.063051	3.688023	23.963	U	17.606	19.038	U
Ghent	East Flanders	1.4	350	490	51.05747778	3.716	20.386	U	25.321	12.737	U
Herzele	East Flanders	1	220	220	50.89567	3.846882	6.472	SU	5.460	18.236	SU
Merelbeke	East Flanders	1.3	95	123.5	50.96004444	3.718902778	6.229	SU	4.030	19.239	SU
Oudenaarde	East Flanders	1.7	200	340	50.84445	3.614216667	2.453	R	8.810	20.725	SU
Kortrijk	West Flanders	0.7	4.5	3.15	50.812226	3.249082	14.808	U	15.069	7.001	U
Roeselare	West Flanders	1	330	330	50.951716	3.130662	20.265	U	16.387	1.594	U
Abdijvhpark	Flemish-Brabant	0.55	18000	16500	50.86362222	4.723430556	0.030	SU	13.188	15.164	U
Bierbeek	Flemish-Brabant	1.6	40	64	50.835946	4.787318	6.152	SU	2.512	16.494	SU
Kerkom	Flemish-Brabant	0.1	250	25	50.83590278	4.907333333	9.816	SU	3.277	12.119	SU
Leuven	Flemish-Brabant	1	590	590	50.86673333	4.698511111	19.391	U	13.743	22.061	U
Meerdaal	Flemish-Brabant	1	60	60	50.80876944	4.724988889	0	R	1.430	60.872	R
Overijse	Flemish-Brabant	1	875	875	50.79711111	4.549427778	4.760	R	4.178	28.692	R
Rotselaar	Flemish-Brabant	0.15	860	129	50.961586	4.708369	10.991	U	3.838	33.169	R
Rotselaar-gre	Flemish-Brabant	1	275	275	50.96725278	4.735088889	0	R	4.857	36.679	R
Zoetwater	Flemish-Brabant	0.2	15625	3125	50.822919	4.653563	0.240	R	2.333	45.184	R

Table S2: Monthly averages for time of sunrise and sunset used to calculate daytime and nighttime average, minimum and maximum temperatures throughout the year.

Month	Sunrise	Sunset
January	08:30	17:15
February	08:00	18:00
March	07:30	19:15
April	07:00	20:30
May	06:00	21:30
June	05:30	22:00
July	05:45	21:45
August	06:30	21:00
September	07:15	20:00
October	07:30	18:30
November	08:00	17:00
December	08:30	16:45

Table S3. Results of the linear mixed-effect models on mean daytime temperature (°C) and daytime temperature range (Δ °C)**.** Significant results ($p < 0.05$) are given in bold .

	Mean daytime temperature (°C)			**Daytime temperature range (Δ °C)**		
Fixed effects	**F**	**df**	**p**	**F**	**df**	**p**
Urbanization level 50 m	**18.434**	**2**	**< 0.001**	**11.9285**	**2**	**0.002**
Urbanization level 3200 m	0.029	2	0.985	2.759	2	0.252
season	**281.322**	**3**	**< 0.001**	**556.408**	**3**	**< 0.001**
Depth	2.332	1	0.127	1.376	1	0.241
Area	2.3374	1	0.126	0.642	1	0.423
Day	**281.795**	**6**	**< 0.001**	**192.037**	**6**	**< 0.001**
Urbanization level 50 m x season	**51.700**	**6**	**< 0.001**	**451.078**	**6**	**< 0.001**
Urbanization level 3200 m x season	**18.434**	**2**	**< 0.001**	**11.928**	**2**	**0.002**

Table S4. Results of the linear mixed-effect models on mean nighttime temperature (°C) and nighttime temperature range (Δ °C). Significant results ($p < 0.05$) are given in bold.

	Mean nighttime temperature (°C)			**Nighttime temperature range (Δ °C, NTR)**		
Fixed effects	**F**	**df**	**p**	**F**	**df**	**p**
Urbanization level 50 m	**22.138**	**2**	**< 0.001**	**15.658**	**2**	**< 0.001**
Urbanization level 3200 m	0.177	2	0.915	5.753	2	0.0563
season	**1239.461**	**3**	**< 0.001**	**263.505**	**3**	**< 0.001**
Depth	**4.417**	**1**	**0.035**	2.911	1	0.088
Area	**3.943**	**1**	**0.047**	0.370	1	0.543
Night	**975.319**	**6**	**< 0.001**	**141.895**	**6**	**< 0.001**
Urbanization level 50 m x season	**190.016**	**6**	**< 0.001**	**480.095**	**6**	**< 0.001**
Urbanization level 3200 m x season	**22.138**	**2**	**< 0.001**	**15.658**	**2**	**< 0.001**

Part 2: Urban Evolution

Chapter 2

The heat is on: genetic adaptation to urbanization mediated by thermal tolerance and body size

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Abstract

Worldwide, urbanization leads to tremendous anthropogenic environmental alterations, causing strong selection pressures on populations of animals and plants. Although a key feature of urban areas is their higher temperature ("urban heat islands"), adaptive thermal evolution in organisms inhabiting urban areas has rarely been studied. We tested for evolution of a higher heat tolerance (CT_{MAX}) in urban populations of the water flea *Daphnia magna*, a keystone grazer in freshwater ecosystems, by carrying out a common garden experiment at two temperatures (20 °C and 24 °C) with genotypes of 13 natural populations ordered along a well-defined urbanization gradient. We also assessed body size and haemoglobin concentration to identify underlying physiological drivers of responses in CT_{MAX}. We found a higher CT_{MAX} in animals isolated from urban compared to rural habitats and in animals reared at higher temperatures. We also observed substantial genetic variation in thermal tolerance within populations. Overall, smaller animals were more heat tolerant. While urban animals mature at smaller size, the effect of urbanization on thermal tolerance is only in part caused by reductions in body size. Although urban *Daphnia* contained higher concentrations of haemoglobin, this did not contribute to their higher CT_{MAX}. Our results provide evidence of adaptive thermal evolution to urbanization in the water flea *Daphnia*. In addition, our results show both evolutionary potential and adaptive plasticity in rural as well as urban *Daphnia* populations, facilitating responses to warming. Given the important ecological role of *Daphnia* in ponds and lakes, these adaptive responses likely impact food web dynamics, top-down control of algae, water quality, and the socio-economic value of urban ponds.

INTRODUCTION

The ongoing exponential growth of human populations will continue to lead to an increase in the number of cities, their size, and the expansion of the total urban area worldwide (Angel *et al.*, 2011). By 2050, 66% of the world's population is expected to live in urban settlements (UN, 2014). Urban areas are hotspots of anthropogenic activity, concentrating environmental alterations and shaping biotic interactions as a result of a changed hydrology, atmospheric chemistry, microclimate, nutrient load, land use, pollution, vegetation cover, and the introduction of non-native species (Kaye *et al.*, 2006; Seto *et al.*, 2010). Consequently, the persistence of populations and communities of many animal and plant species is challenged in urban areas, and cities impose strong selection factors on biota (McKinney, 2008; Alberti, 2015) .

Evolutionary changes can take place at the same spatio-temporal scale as ecological processes, in a dynamic interplay (i.e. 'eco-evolutionary dynamics', Hendry, 2017). This interaction between ecological and evolutionary trait change can be especially important in rapidly changing, novel environments, such as the extremely dynamic urban areas. There is an increasing number of studies reporting remarkable genetic differentiation among populations of several species along urban-rural gradients and evolutionary trait change within populations in urban settlements (Donihue & Lambert, 2015; McDonnell & Hahs, 2015; Tüzün *et al.*, 2015; Brans *et al.*, 2017a). For example, as a response to habitat fragmentation and warming, plants in urban areas show reduced dispersal (Cheptou *et al.*, 2008), bolt early, grow larger, have a reduced number of leaves and produce more seeds (Yakub & Tiffin, 2016). One of the most distinct consequences of urbanization and a clear example of anthropogenic microclimate modification is the occurrence of a local-scale temperature gradient, known as the 'urban heat island' effect (UHI, Oke, 1973; Ward *et al.*, 2016). This well-studied phenomenon is defined by an increased temperature in city centers, gradually declining towards the suburban fringe and rural areas. UHIs are mainly the result of urban geometry, thermal properties of the materials used in urban planning, and a high human energy consumption and heat output (Santamouris, 2007; Hart & Sailor, 2009). As a result, species preferring and adapted to warm and dry habitats are overrepresented in cities (Knapp *et al.*, 2008; Duffy & Chown, 2016; 2010; Piano *et al.*, 2017).

Temperature is one of the most crucial environmental conditions shaping organismal fitness (Angilletta, 2009). Yet, studies on thermal tolerance and performance of organisms along urbanization gradients are rare and focus on terrestrial organisms (McLean *et al.*, 2005; Angilletta *et al.*, 2007; Diamond *et al.*, 2017b) . Moreover, these studies mostly involve phenotypic trait shifts as observed in field collected animals (Angilletta *et al.*, 2007; Kaiser *et al.*, 2016). For example, Angilletta *et al.* (2007) reported higher knock-down resistance (time of immobilization when exposed to an extreme temperature) in field-caught city ants compared to rural ants, indicating adaptive responsiveness in thermal tolerance to rapid microclimatic changes. Such studies on field-collected animals, however, do

not allow separating genetic from plastic responses. To understand how populations respond to the urban-rural gradient it is important to quantify the relative importance of the genetic, plastic and genotype × environment interaction component of trait change (Ghalambor *et al.*, 2007; Levis & Pfennig, 2016), and to assess to what extent populations harbour quantitative genetic variation for thermal tolerance and thus show the capacity to evolve in the future (Gunderson & Stillman, 2015; Diamond, 2017). McLean *et al.* (2005) found evolution of thermal performance curves of growth rate in urban soil fungi, grown along a temperature gradient in a common-garden setting. So far, however, in urban populations of animals there is little evidence of genetic adaptation for thermal tolerance, measured as the critical thermal maximum (CT_{MAX}) or time to immobilization at higher temperatures (Huey *et al.*, 1992; Angilletta, 2009). CT_{MAX} is defined as the thermal endpoint at which locomotory functions are compromised and the organism faints (Huey *et al.*, 1992; Lutterschmidt & Hutchison, 1997), and is a common metric used as a proxy for thermal tolerance (e.g.; Rezende *et al.*, 2011; Overgaard *et al.*, 2012; Geerts *et al.*, 2015). Diamond *et al.* (2017b) recently reported a higher CT_{MAX} for urban compared to rural acorn ants. While their results were strongly suggestive, their experimental design did not allow to entirely differentiate between maternal effects and genetic differentiation.

Aquatic ectotherms are especially prone to be affected by anthropogenic warming, as they cannot actively regulate their body temperature, and oxygen supply is limited in aquatic systems (Zuo *et al.*, 2012; Gunderson & Stillman, 2015; Verberk *et al.*, 2016). Whereas metabolic rates, and thus oxygen demands, rise with increasing temperatures, oxygen solubility in the water declines. This induces additional physiological stress on aquatic organisms upon warming (Atkinson, 1994; Angilletta *et al.*, 2004; Daufresne *et al.*, 2009). In the present study, we tested whether populations of the water flea *Daphnia magna* have adapted to urban heat island effects through an increased thermal tolerance compared to rural populations. *Daphnia magna* is a well-studied species and an ecologically strong interactor in aquatic habitats (Miner *et al.*, 2012). It has recently been shown that populations of this species can and have evolved in their thermal tolerance in response to climate change (Geerts *et al.*, 2015). We performed a common garden experiment using genotypes isolated from ponds along a well-defined urban-rural gradient cultured at 20 °C and 24 °C, and scored for their critical thermal maximum. Urbanization was assessed by calculating the percentage built-up area at a radius of 3200 m around the pond. Given that percentage built-up area includes surface area occupied by buildings and industrial infrastructure, but excludes roads and parking lots, a built-up area of 10% and more is considered highly urbanized. To explore the role of two possible underlying processes driving thermal tolerance, we also quantified body size and haemoglobin (Hgb) concentrations in all genotype x environment combinations (Williams *et al.*, 2011; Klockmann *et al.*, 2017;). We previously found a significant size reduction in *D. magna* along the studied urbanization gradient (Brans *et al.*, 2017a), in line with the urban heat island effect (cf. 'temperature-size rule'; Atkinson, 1994). Size reductions are a powerful way for organisms to cope with physiological stress associated with increased respiration and metabolic rate, and the

reduced solubility of oxygen with higher temperatures (Daufresne *et al.*, 2009). Yet, size reduction comes at a cost, because, amongst others, body size in *Daphnia* is related to competitive strength and grazing capacity (Tessier *et al.*, 2000; Gianuca *et al.*, 2016). This size-mediated trade-off between competitive strength and heat tolerance (Kelly *et al.*, 2013) may lead to the evolution of increased thermal tolerance through physiological mechanisms, independent of body size reduction. To the extent that thermal tolerance is related to oxygen supply (Pörtner, 2001), one possible mechanism may be an increase in haemoglobin concentrations (Paul *et al.*, 2004; Seidl *et al.*, 2005). We thus quantified evolution and phenotypic plasticity of thermal tolerance (CT_{MAX}) as well as body size and haemoglobin concentration along the urbanization gradient. We furthermore explored the possible mediating role of both body size and haemoglobin concentrations in upregulating thermal tolerance along the gradient and at higher rearing temperatures.

MATERIAL AND METHODS

Study organism and sampling sites

Daphnia magna clones originated from 12 ponds (i.e. 12 populations) along an urban-rural gradient in Flanders (Belgium), sampled during the summer of 2013 (Fig. S1, Supplementary Information; see also Brans *et al.*, 2017a). One additional population was collected in the highly urbanized city center of Brussels during August 2015 (*Brxl*, Fig. S1). The degree of urbanization was calculated based on the percentage built-up area within a radius of 3200 meter around each pond, derived from LRDgis (Large-scale Reference Database) maps using Ground building values obtained from the Flanders Geographical Information Agency. Percentage built-up area, i.e. surface area occupied by buildings, houses and industrial infrastructure, with roads and parking lots excluded, is considered high from 10% upwards. Information on pond location and percentage built-up area can be found in Table S1 (Supplementary Information).

Six individuals per population were isolated to start clonal cultures. *D. magna* has a cyclical parthenogenetic reproduction cycle and reproduces asexually under favorable environmental conditions, producing all-female clonal populations. Their clonal identity was screened by means of 27 microsatellite markers, structured in four multiplexes, following Jansen *et al.* (2011b) and Orsini *et al.* (2012). Details on the genotyped markers, multiplex structure, genomic DNA extraction (according to Mergeay *et al.*, 2008), PCR cycling conditions, and microsatellite scoring are given in Supplementary Information. The scored microsatellite loci are evenly distributed across the chromosomal map of *D. magna* (Routtu *et al.*, 2010). All lines that were differentiated in at least one of the microsatellite loci were considered independent clonal lineages (i.e. 'clone') in the statistical analyses (see also Table S2, Supplementary Information).

Experimental set-up and response variables

Maternal effects and culture conditions

To obviate interference from (grand)maternal effects, six replicate cultures (two temperature treatments x three replicates, see *Overall design*) of each line were established (i.e. 36 cultures per population) and cultured independently for at least two generations. Individual *Daphnia* were reared in dechlorinated tap water under standardized laboratory conditions (temperature-controlled water baths set at 20 °C, 14:10h L:D photoperiod). Eighty percent of the medium was refreshed every other day and animals were daily fed *Acutodesmus obliquus* green algae (0.8×10^5 cells ml^{-1} for juveniles and $1x10^5$ cells ml^{-1} for adults). To avoid crowding, newborn juveniles were removed. For the additional population (*Brxl*, see *Study organism and sampling sites)*, only four lines (each being a distinct clonal lineage, with one replicate per lineage) were included, and the animals were cultured for only one generation under standardized conditions prior to the experimental temperature exposure. Because of this less stringent standardization, we present results both with and without inclusion of this population.

Overall design

To test for effects of the urbanization gradient and rearing temperature on thermal tolerance we set up a full factorial experiment with 13 populations x 6 lines x 2 rearing temperatures (20 °C and 24 °C, *Brxl* only 4 lines). Each combination was replicated three times (*Brxl* one replicate) resulting in 440 experimental units. Per replicate, a cohort of 12 experimental neonates (< 24h old, 2nd up to 4th clutch) was transferred into a 500ml jar containing dechlorinated tap water in a temperature-controlled water bath at 20 (± 0.6) °C or 24 (± 0.4) °C with a 14:10 L:D photoperiod. Animals were cultured as described above. Upon maturity, thermal tolerance was scored on six first adult instar individuals of each cohort, four of which were also used to assess size at maturity (body size). Both CT_{MAX} values and body size measurements were averaged for each cohort and used as input data. We quantified haemoglobin concentration on the remaining six individuals per replicate cohort in their third adult instar. Body size was measured on three animals and converted to dry mass using the *D. magna* length-mass equation described in Dumont *et al.* (1975). The pooled set of six animals was then stored at -80 °C for later assessment of haemoglobin concentrations.

Critical Thermal Maximum (CT_{MAX})

To determine CT_{MAX}, the temperature at which *D. magna* fails to maintain motoric functioning, we applied the method optimized by Geerts *et al.* (2015). Of each cohort, six random individuals in their first adult instar were individually transferred into a 0.5 ml Eppendorf tube. Four of these animals were measured (Olympus SZX12, Olympus optical co., LTD, from the eye to the base of the tail spine) to determine the average body size at the time of the CT_{MAX} measurement (i.e. size at maturity) for each cohort. The six Eppendorf tubes were then randomly placed into the central section of a 4 x 6 thermal heater (Peqlab Digital Block Heater HX-2). Animal activity was continuously observed during the

heating process, which started at 20 °C and gradually increased in steps of 1 °C per 20 (± 5) seconds. This heating rate yields highly repeatable data on thermal tolerance in *Daphnia* and allowed detecting subtle thermal evolution of CT_{MAX} between *D. magna* subpopulations separated in time (Geerts *et al.*, 2015). CT_{MAX} was scored by monitoring the temperature at which the animal stops swimming and sinks to the bottom of the Eppendorf tube; effectiveness was optimized using voice recording. After fainting, individuals were immediately transferred to ambient conditions (20 °C) to confirm recovery (seven out of 2640 individuals did not recover, their CT_{MAX} score was left out of the analysis). CT_{MAX} measurements of all six individuals per experimental unit were averaged, leading to one CT_{MAX} value for each treatment x clone x replicate. Replicate CT_{MAX} measurements of all treatment x population cohorts were randomized in time.

Haemoglobin (Hgb) concentration

Haemoglobin concentration, an important parameter linked to oxygen transport and homeostasis (Lamkemeyer *et al.*, 2003; Paul *et al.*, 2004; Zeis *et al.*, 2004), and known to induce higher thermal tolerance in *Daphnia* (Paul *et al.*, 2004; Seidl *et al.*, 2005), was spectrophotometrically assessed following Yampolsky *et al.* (2014); details are given in Supplementary Information. Due to accidental thawing, *Brxl* samples could not be processed for this variable. The total haemoglobin concentration (further referred to as Hgb) was calculated as the relative peak height at 576 nm using the formula $H_{576} = A_{576} - (A_{560} + A_{600}) / 2$, based on the recorded absorbance at these wave lengths, and standardized by dry mass.

Statistics

We tested for effects of urbanization level (continuous, expressed as percentage built-up area, log-transformed) and rearing temperature (categorical; 20 °C and 24 °C) on the two response variables (CT_{MAX} and total Hgb concentration) using separate linear mixed-effect models. The statistical tests were performed in R, version 3.2.3. (R Development Core Team, 2015). The 'lme4' package was used for model fitting and the 'car' package to compute approximate F-test statistics and p-values for fixed effects. Size at maturity was included as a covariate when analyzing CT_{MAX}. *Daphnia* mature at a smaller size in more urban ponds (Brans *et al.*, 2017a) and smaller daphnids have a higher CT_{MAX} (Geerts *et al.*, 2014; 2015; see also Fig. S2, Supplementary Information). We incorporated the day on which CT_{MAX} was scored as a random effect and checked for a possible effect of pond depth by adding it as a covariate in the analysis. Pond depth was, however, not found to impact CT_{MAX} significantly ($F_{1, 10.26} = 3.041$, $p = 0.111$) and was removed. Clone (nested in population) and population were included as random effects in all models. We applied the restricted maximum likelihood estimation method and corrected the degrees of freedom for fixed effects using the Kenward-Roger approximation (Alnosaier, 2007). All model assumptions were visually checked (plotting histograms of model residuals, residuals versus fitted values, and normal Q-Q plots) and we formally tested for deviations from normality by using the

Shapiro-Wilk test, and for heterogeneity of variances with temperature treatment as a grouping factor using Levene's test. Model assumptions were met when analyzing CT_{MAX}. Hgb values were log-transformed to better meet assumptions of normality. Significance of random effects was tested by means of model comparison (Wald's Chi-square values and p-values were computed using the 'stats' package).

To quantify the direct effects of urbanization level and rearing temperature on thermal tolerance (CT_{MAX}), together with possible indirect relationships working through Hgb concentration and body size, we performed piecewise structural equation modeling (Shipley, 2009; Lefcheck, 2016; Sprau *et al.*, 2016) using the packages 'nlme' and 'piecewiseSEM' (Lefcheck, 2016) in R. This confirmatory path analysis method enables to test the overall fit of a multifaceted network of causality (Grace, 2006), including the estimation of indirect effects, while allowing the use of a nested data structure and random effects (Shipley, 2009). To derive comparable estimates, all quantitative predictors and CT_{MAX} were standardized to a mean of zero and standard deviation of one. Based on the literature (see legend of Fig. S3, Supplementary Information), we constructed an *a priori* model with hypothesized pathways between urbanization level (U, log-transformed) and temperature (T) treatment (both exogenous variables, i.e. variables of which variation is a result of components outside the model) and size, Hgb concentration (Hgb, log-transformed to improve data normality), and CT_{MAX} (all being endogenous variables, i.e. variables of which the variance is to be explained by the model). The associated path diagram is depicted in Fig. S3 (Supplementary Information). We applied d-separation tests (d-sep; Shipley, 2009) to evaluate overall path models, to check the significance of non-hypothesized independent pathways, and to validate whether inclusion of significant missing path(s) improved our models. Model acceptance was based on the Fisher's C statistic, with a model being accepted if the associated $p > 0.05$ (Shipley, 2009). We here report the final accepted model, accompanied by conditional R^2 values (R^2_c, all factors included) for general linear-mixed effect models. Missing pathways generated by this model were found to be not significant and therefore not included. Proportional contributions of significant direct and indirect (via body size and Hgb) pathways to the change in thermal tolerance due to urbanization, temperature and the interaction term between both (expressed in units of standard deviation) were calculated using the standardized path coefficients based on Grace & Bollen (2005).

RESULTS

Critical Thermal Maximum (CT_{MAX})

Thermal tolerance, measured as CT_{MAX}, increased with the level of urbanization and was higher at the higher (24 °C) than at the lower (20 °C) rearing temperature (Table 1a, Fig. 1). CT_{MAX} was negatively correlated with body size (size at maturity; Fig. S2, Supplementary Information). No significant interactions were found between the pairwise combinations of explanatory variables, or the three-way interaction (Table 1a). Model comparison showed a significant effect of the random effects date and clone, the latter indicating genetic variation for CT_{MAX} within populations (Table 1b). Excluding *Brxl* from the analysis gave qualitatively the same results (Table S3, Fig. S4, Supplementary Information).

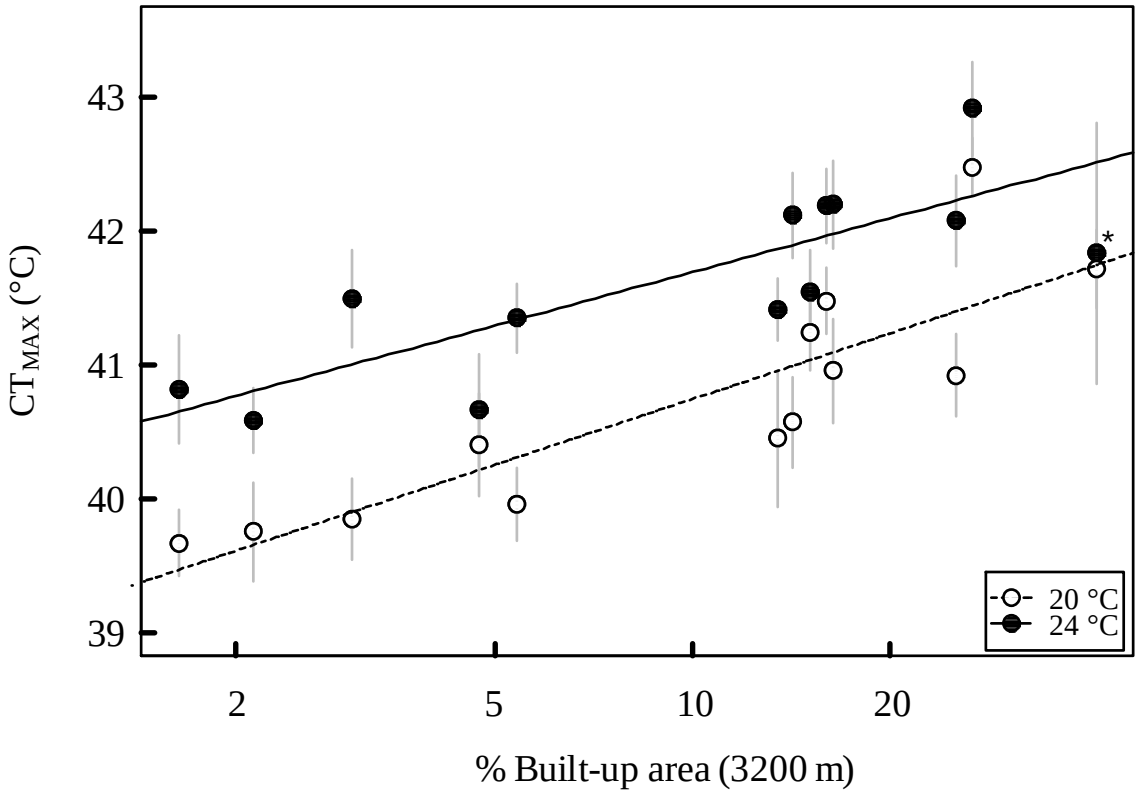


Figure 1. Average critical thermal maxima plotted against the level of urbanization for all 13 *Daphnia magna* populations. Given are population means ±1 SE when reared at 20 °C (open symbols; dashed line) and at 24 °C (filled symbols; full line). The urbanization level is plotted as percentage built-up area (+ 1, log-scale) within a radius of 3200 m around each sampling location. Regression lines are given for both rearing temperatures based on the slope in the mixed model. Analysis without the Brxl population (*) gave qualitatively the same results (see Supplementary Information).

Total haemoglobin (Hgb) concentration

Total Hgb concentration increased significantly along the urbanization gradient, but was not affected by the rearing temperature (Table 1a, Fig. 2). Model comparison showed no significant influence of random effects (Table 1b).

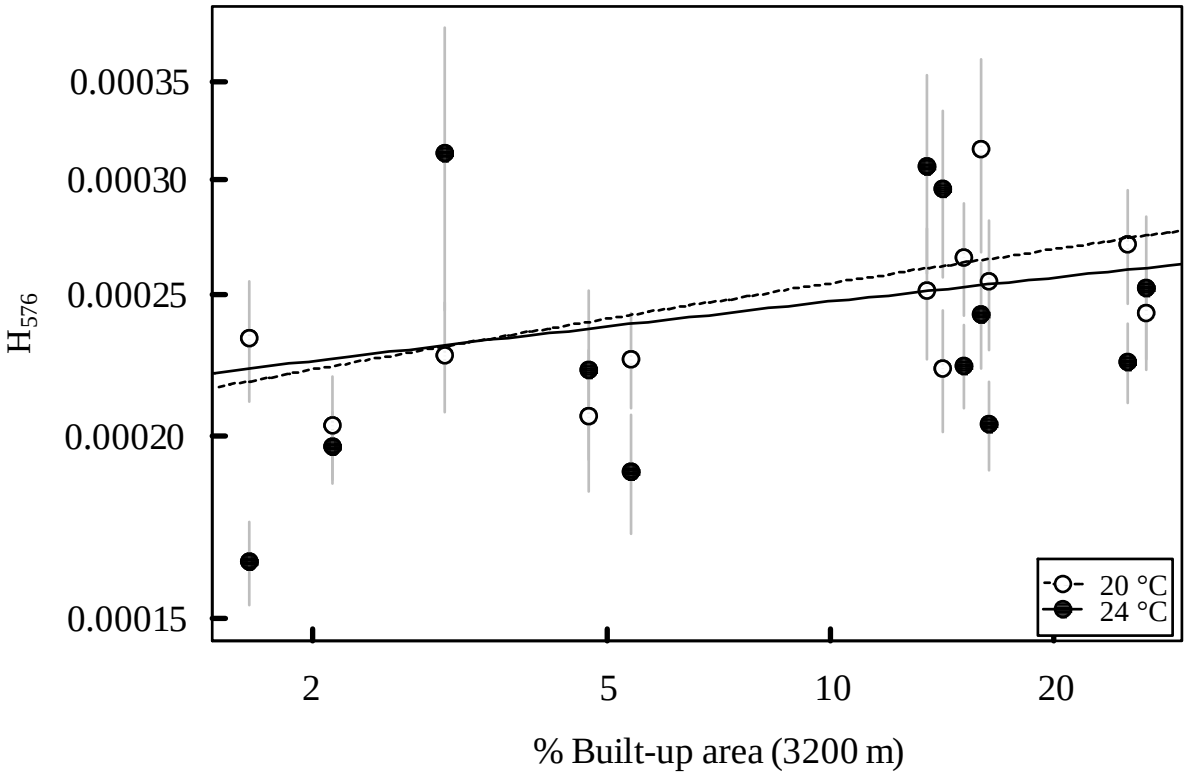


Figure 2. Total haemoglobin concentration plotted against the level of urbanization for 12 *D. magna* populations (with exception of Brxl). Population means ± 1 SE (H576, relative haemoglobin absorption peak height at 576 nm, weighted by lg dry mass) are given when reared at 20 °C (open symbols; dashed line) and at 24°C (filled symbols; full line) and plotted on a log-scale. The urbanization level is plotted as percentage built-up area (+1, log-scale) within a radius of 3200 m around the sampling location. Regression lines are given for both rearing temperatures based on the slope in the mixed model.

Confirmatory path analyses

The final path model was strongly supported by the data (Fisher's C = 0.76, p = 0.993; depicted in Fig. 3). It revealed a direct positive effect of both urbanization level and rearing temperature on thermal tolerance (β ± SE, urbanization: 0.303 ± 0.053, p < 0.001; temperature: 0.567 ± 0.081, p < 0.001; R^2_c thermal tolerance = 0.788). Moreover, both exogenous variables also indirectly elevated thermal tolerance by inducing a smaller size at maturity (β ± SE, urbanization: -0.519 ± 0.116, p = 0.001; temperature: -0.245 ± 0.076, p < 0.001; R^2_c size = 0.418). While urbanization also increased Hgb content (β ± SE = 0.180 ± 0.056, p = 0.009), this did not contribute to a higher thermal tolerance because the Hgb concentration did not affect thermal tolerance (p = 0.419).

The proportional contribution of urbanization-driven size reductions to the estimated total effect of urbanization and temperature on thermal tolerance was 10.85%, whereas the direct effect of urbanization contributed 27.79% (Fig. 3b). Size-mediated temperature effects on thermal tolerance contributed only 5.12%, while the direct effect of rearing temperature on thermal tolerance accounted for 52.00%. Overall, urbanization level (reflecting evolutionary trait change, Fig. 3b) and rearing temperature (reflecting phenotypic plasticity, Fig. 3b) accounted for 38.64% and 57.11% of the total increase in CT_{MAX}, respectively.

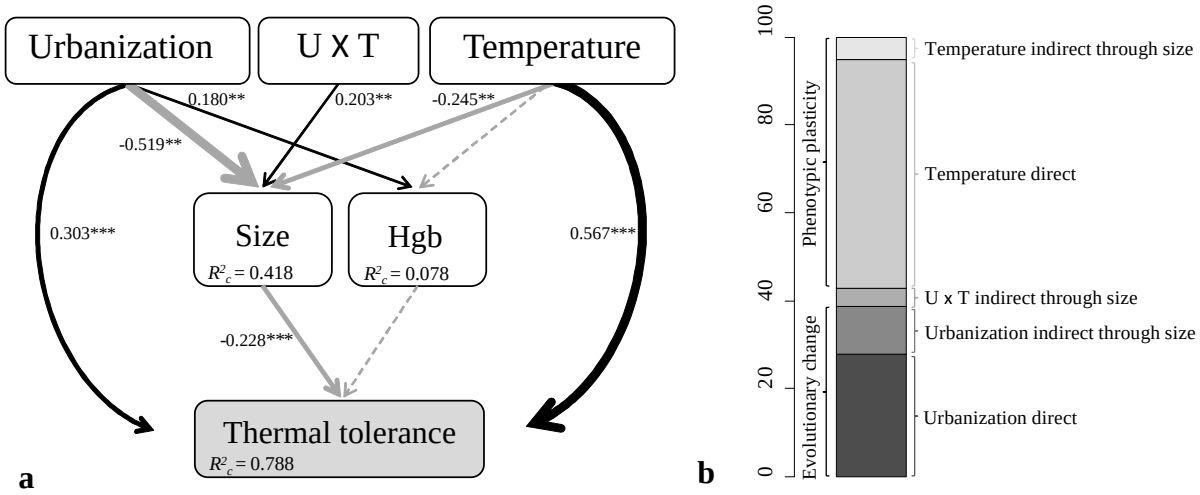


Figure 3. Final path model on how urbanization level and rearing temperature directly and indirectly (through effects on body size and haemoglobin concentration, Hgb) shape thermal tolerance in *D. magna*. (a) Path diagram with standardized path coefficients, reported next to the arrows, and R^2 values (R^2_c, including all factors) given for all response variables. Black and grey solid arrows indicate significant positive and negative pathways, respectively, derived from piecewise SEM. Thickness of arrows is proportional to the standardized path coefficients. Non-significant pathways are given in dashed grey lines. Significance levels are indicated according to: ***p < .001; **p < .01; *p < .05. (b) Proportional contribution of significant indirect (via size) and direct pathways to the estimated total effect of urbanization (i.e. "evolutionary change"), temperature (i.e. "phenotypic plasticity"), and their interaction term (U x T), on thermal tolerance, calculated using the standardized path coefficients (following Grace & Bollen, 2005).

Table 1. Results of the linear mixed-effect models for CT_{MAX} and total haemoglobin concentration for the (a) fixed effects and the (b) random effects. Size at maturity was included as covariate in the CT_{MAX} analysis. Haemoglobin values were log-transformed. Significant p-values ($p < 0.05$) are given in bold.

a) Fixed effects	**CT_{MAX}**				**Total haemoglobin concentration**			
	F	**Df**	**Df_{res}**	**p**	**F**	**Df**	**Df_{res}**	**p**
Urbanization level	30.070	1	13.32	**<0.001**	10.261	1	9.10	**0.011**
Temperature	73.332	1	331.50	**<0.001**	1.694	1	354.45	0.194
Size (at maturity)	4.615	1	332.59	**0.032**				
Urbanization x Temperature	0.016	1	334.02	0.898	0.002	1	355.34	0.966
Urbanization x Size	0.048	1	294.70	0.827				
Temperature x Size	0.043	1	370.66	0.835				
Urbanization x Temperature x Size	1.003	1	335.02	0.317				

b) Random effects	**CT_{MAX}**			**Total haemoglobin concentration**		
	χ^2	**Df**	**p**	**χ^2**	**Df**	**p**
Population	0.017	1	0.896	0.001	1	0.974
Clone	5.730	1	**0.017**	1.241	1	0.265
Day	64.454	1	**<0.001**			

DISCUSSION

We here show a distinct evolutionary response towards higher thermal tolerance in *Daphnia magna* populations inhabiting urban ponds, reflecting adaptive genetic trait divergence associated with the urban heat island effect. Moreover, a change in rearing temperature of + 4 °C triggered a substantial acclimatory increase in heat tolerance, indicating adaptive plasticity for this trait. We also observed genetic changes in body size and haemoglobin concentrations along the same gradient, and showed that urban-driven size reduction indirectly augmented heat tolerance. Our results reveal pronounced adaptive phenotypic and genotypic trait change in aquatic organisms shaped by urbanization and associated temperature increases, and improve our understanding of organismal responses to future warming due to increased urbanization and climate change.

Urbanization is one of the most profound drivers of current environmental change (Kaye *et al.*, 2006; Grimm *et al.*, 2008a,b) and is rapidly increasing on a global scale (Seto *et al.*, 2012). Although recent studies have reported rapid evolution in urban environments (Atwell *et al.*, 2012; Donihue & Lambert, 2015; Yakub & Tiffin, 2016), remarkably little is known on thermal performance and tolerance of organisms inhabiting urban areas. Only three empirical studies report on urban thermal performance and tolerance, all focusing on terrestrial taxa (McLean *et al.*, 2005; Angilletta *et al.*, 2007; Diamond *et al.*, 2017b). Angilletta *et al.* (2007) documented an increase in phenotypic thermal tolerance in city ants, whereas McLean *et al.* (2005) reported genetic thermal specialization and countergradient variation in two species of urban soil fungi. We here tested for an effect of urbanization on genotypic trait divergence in three key traits linked to thermal physiology, i.e. heat tolerance (CT_{MAX}), haemoglobin concentration (Hgb), and body size, in *D. magna*, a poikilothermic aquatic filter feeder.

In agreement with earlier studies on *D. magna* thermal tolerance (Lamkemeyer *et al.*, 2003; Paul *et al.*, 2004; Williams *et al.*, 2011; Yampolsky *et al.*, 2014), we found that higher rearing temperature (24 °C) on average induces an increase in thermal tolerance of ca. 1.25 °C compared to 20 °C, reflecting adaptive plasticity. Our results also show that urban populations have a CT_{MAX} of up to 2 °C higher than their rural counterparts. These findings confirm our hypothesis that *Daphnia* genetically adapts to the presence of urban heat islands and associated extreme temperature events (Christidis *et al.*, 2015; De Ridder *et al.*, 2015; Ward *et al.*, 2016). De Ridder *et al.* (2015) reported UHI effects ranging between 1.5 °C and 4.5 °C with extremes of up to 8°C difference in average summer mean surface temperature between rural and urban sites in Flanders over the period 2001-2013. Average and maximum summer day temperature, measured during the period of July-August 2014 in two urban and two rural ponds, differed ca. 4 °C ($\Delta T[°C]_{urb\text{-}rur}$; Brans *et al.*, 2017a). Assuming an average UHI of + 4 °C and summer averages of daily maximum temperatures in rural ponds being approximately 20 °C (Brans *et al.*, 2017a; De Ridder *et al.*, 2015), the combination of genotypic (urban versus rural populations) and phenotypic (acclimatization to 24 °C compared to 20 °C) trait change in the *D. magna*

populations studied by us would lead to a CT_{MAX} increase of ca. 3.25 °C from rural to urban populations. Given that CT_{MAX} reflects tolerance to extreme temperatures, its evolutionary increase is most likely not only driven by increased average temperatures, but also triggered by heatwaves, which are more common in cities (Ward *et al.*, 2016). These results are in line with recent results on community responses to urbanization in invertebrates (Kaiser *et al.*, 2016; Piano *et al.*, 2017) and suggest that the studied *D. magna* populations are quite well equipped to cope with the observed urban-driven warming of habitats in Flanders. Piano *et al.* (2017) showed that urban communities of carabid beetles in Flanders (including the same cities and study area as the present study) are characterized by a higher number of thermophilic species and a reduced richness of species with low thermal preferences, causing a shift in thermal profile at the community level..

Our observation of a pronounced evolutionary response in thermal tolerance to urbanization reinforces earlier evidence on rapid evolution of CT_{MAX} in *Daphnia* in response to temperature increases in nature and in experimental evolution (Geerts *et al.*, 2015). Geerts *et al.* (2015) showed a significant evolutionary increase (+1°C) in heat tolerance in a population of *D. magna* in response to climate change by comparing the thermal tolerance of clonal lineages hatched from dormant eggs deposited in the 1960s with that of clonal lineages hatched from dormant eggs deposited in the 2000s; a period over which the region where the lake was situated experienced a temperature increase of 1.15 °C. The same study revealed that clones hatched from dormant eggs deposited in outdoor mesocosms that were exposed to ambient temperature + 4 °C during two years showed a CT_{MAX} that was 3.5 °C higher than clones hatched from dormant eggs of populations exposed to ambient temperature (control treatment; Geerts *et al.*, 2015). Together with these previous findings, our results provide solid proof that *Daphnia* can evolutionary track environmental warming, not only along large-scale temperature gradients (Geerts *et al.*, 2014; Williams *et al.*, 2011; Yampolsky *et al.*, 2014) or through time (Geerts *et al.*, 2015), but also along smaller scale spatial disturbance gradients such as the urbanization gradients studied here. Urban areas have recently been accredited as being 'natural experiments' that can provide important information to understand organismal responses to future climate change (Grimm *et al.*, 2008a,b; Youngsteadt *et al.*, 2015). Our results reveal parallel pathways of evolutionary and plasticity responses to both climate change (cf. resurrection ecology study in Geerts *et al.*, 2015) and urbanization (present study) scenarios. In addition, we found substantial genetic variation for CT_{MAX} in all populations along the gradient, reflecting evolutionary potential in rural as well as urban populations to respond to future warming, either in response to urbanization, global warming, or a combination of both (Diamond, 2017). Our results indeed suggest that rural populations can likely adapt to a 4 °C temperature increase and the associated occurrence of extreme temperature events projected to occur over the next 100 years under the IPCC RCP8.5 climate change scenario (UN, 2014).

Using structural equation modelling, we revealed both direct and indirect pathways leading to the observed increased heat tolerance in *D. magna* along the urbanization gradient (i.e. reflecting

evolutionary change) and when cultured at a higher temperature (i.e. phenotypic plasticity). Genetic differentiation among populations along the urbanization gradient accounted for almost 39% of the combined genotypic and phenotypic increase in CT_{MAX}, whereas 57 % was linked to the temperature at which the animals were cultured. Urban *Daphnia* achieve a higher CT_{MAX} than rural ones in part through a reduction in body size. Maturing at a smaller size is a common strategy for animals that are exposed to increased temperatures, as it enhances their capacity to cope with elevated oxygen demands as metabolic rates increase (Magura *et al.*, 2006; Liker *et al.*, 2008; Daufresne *et al.*, 2009; Meillère *et al.*, 2015). Brans *et al.* (2017a) previously reported a genetic reduction in size at maturity with increased urbanization, which is now shown to feed back to a size-mediated increase in heat tolerance. In addition, *Daphnia* from ponds in highly urbanized areas exhibit a higher CT_{MAX} than similarly sized *D. magna* individuals from more rural habitats. Overall, the size-independent evolutionary increase in CT_{MAX} contributed more than double (72%) to the total evolutionary change in CT_{MAX} along the urbanization gradient compared to the evolutionary increase in CT_{MAX} that was mediated by body size (28%). The phenotypic plasticity response to higher rearing temperature was nearly exclusively driven by a pure effect of temperature (92%), rather than by a change in body size (8%).

Haemoglobin concentrations increased with increasing urbanization, possibly reflecting a temperature-associated hypoxia cline in combination with a temperature related increase in metabolic rate and thus oxygen demand (Atkinson, 1994; Zuo *et al.*, 2012). Moreover, upregulation of haemoglobin expression can be associated with a higher protection against oxidation damage in *Daphnia* (Kim *et al.*, 2009), which is likely highly relevant in the context of urbanization and its associated increased levels of chemical pollution and other stressors. In contrast to expectations based on published work (Lamkemeyer *et al.*, 2003; Zeis *et al.*, 2004; Yampolsky *et al.*, 2014), we did not observe a direct temperature-induced increase in haemoglobin in animals reared at 24 °C compared with animals reared at 20 °C. We see two potential explanations. First, haemoglobin production is costly (i.e. proteins in general are considered to be the most expensive molecules to produce; Jobling, 1983; Thor, 2000), and temperature-induced oxygen deficiencies in the water can in part be buffered by higher ventilation (movement of thoracal appendages) and circulation rates (Paul *et al.*, 2004). Another possibility lies in our methodological procedures: as medium was renewed every two days, we regularly added oxygen-rich water, and we prevented accumulation of organic material. It is thus possible that in natural settings higher temperatures would result in a plastic increase in haemoglobin levels. In contrast with what has been reported in other studies (Pörtner, 2001; Seidl *et al.*, 2005; Williams *et al.*, 2011), increases in CT_{MAX} in *Daphnia* are not mediated by higher haemoglobin concentrations, linked to increased oxygen delivery. Yampolsky *et al.* (2014) similarly did not find a positive effect of higher haemoglobin concentrations on thermal tolerance within temperature treatments. It is possible that other proteins, such as heat shock proteins, can account for the observed increase in heat tolerance in urban compared to rural populations or upon exposure to 24 °C (Feder & Hofmann, 1999; Tomanek, 2010).

To conclude, we found strong signals of thermal adaptation to urbanization in *D. magna*, which most likely contributes significantly to the establishment and persistence of viable populations of this large key-stone species in urban ponds (Brans *et al.*, 2017a). The observed evolutionary tracking of temperature increase in urban areas can be important for the maintenance of the clear-water state in urban ponds and lakes, and thus for the socio-economic value of urban ponds associated with the clear-water state. Urban ponds are considered crucial local biodiversity hotspots (Hassall, 2014). Large-bodied *Daphnia* such as *D. magna* exert substantial top-down control on algae (Dodson, 1974; Jeppesen *et al.*, 1999) and play an essential role in aquatic food web dynamics (Scheffer *et al.*, 1993). Our findings add to the rapidly increasing evidence of phenotypically plastic and adaptive evolutionary trait change in populations of a wide range of species inhabiting urban areas (Donihue & Lambert, 2015; Tüzün *et al.*, 2015; Yakub & Tiffin, 2016; Alberti *et al.*, 2017a,b). Characterizing these phenotypically plastic and genetic responses will be central to understand organismal responses in the light of urbanization and human-driven climate warming (Urban *et al.*, 2016).

Acknowledgements

We thank two anonymous reviewers for their thoughtful and constructive comments. We thank all partners in the Belspo IAP consortium SPEEDY for their support and help with logistics. We thank Jessie MT Engelen for helping with the sampling and Hans Matheve for the assessment of urbanization levels of all populations at different spatial scales. We thank Ria Van Houdt for processing the haemoglobin assays and Melissa Schepens for culturing the algae. We thank Carla Denis for her contribution to the microsatellite analysis.

Supplementary Information "The heat is on: genetic adaptation to urbanization mediated by thermal tolerance and body size"

SUPPLEMENTARY INFORMATION A: MATERIAL AND METHODS

Genotyping of experimental lineages

To obviate interference from (grand)maternal effects, six replicate cultures (two temperature treatments x three replicates, see *Overall design* main text) of each line were established (i.e. 36 cultures per population) and cultured independently for at least two generations. Their clonal identity was screened by means of 27 microsatellite markers, structured in four multiplexes, following Jansen *et al.* (2011b) and Orsini *et al.* (2012). Genomic DNA extraction was conducted on adult *D. magna* individuals, using the Proteinase K digestion method, as described by Mergeay *et al.*(2008). In brief, live *Daphnia* individuals were fixated in 100% ethanol, after which they were homogenized in 100 µl proteinase K-buffer (16mM $[NH_4]_2SO_4$, 67 mM Tris-HCl, pH 8.8, 0.01% Tween-20, 10% DTT and 0.5mM proteinase K). Samples were incubated overnight at 56 °C, followed by a 10-min. denaturation at 96 °C. Qualitative PCR was performed with the QIAGEN multiplex PCR kit (QIAGEN, Netherlands) using a T1 PCR machine (Biometra, Germany). Cycling conditions (i.e. annealing temperatures, given in Table S2) were multiplex dependent and followed Jansen *et al.* (2011b). Microsatellite alleles were scored with an ABI PRISM 3031 automated sequencer (Applied Biosystems) and analyzed with the Gene Mapper software (size standard Liz 500, Applied Biosystems). The scored microsatellite loci are evenly distributed across the chromosomal map of *D. magna* (Routtu *et al.*, 2010). All lines that were differentiated in at least one of the microsatellite loci were considered independent clonal lineages in the statistical analyses. Specific information on the genotyped markers and multiplex structure can be found in Table S2 of this Supplementary Information.

Haemoglobin (Hgb) concentration

Haemoglobin concentration, an important parameter linked to oxygen homeostasis under environmentally induced hypoxia (e.g. due to increased temperatures; Lamkemeyer *et al.*, 2003; Paul *et al.*, 2004; Zeis *et al.*, 2004) and thermal tolerance (Paul *et al.*, 2004; Seidl *et al.*, 2005), was spectrophotometrically assessed following Yampolsky *et al.* (2014). The pooled sample of animals was homogenized in PBS buffer (50 µl / animal, Phosphate-Buffered Saline, 50mM, pH 7.4) and centrifuged for 8 min. at 13,000 rpm and 4 °C. The absorbance spectrum at 576, 560, and 600 nm of the supernatant was then measured spectrophotometrically (Infinite M200, TECAN) within 20 minutes after centrifugation of the samples (due to accidental thawing *Brxl* samples could not be processed for this variable). PBS was used for blank measurements and commercial Hgb samples as standards (Haemoglobin porcine, Sigma-Aldrich H4131). The total haemoglobin concentration (further referred to as *Hgb* in the main text and Fig. S3 of this Supplementary Information) was calculated as the relative

peak height at 576 nm using the formula $H_{576} = A_{576} - (A_{560} + A_{600}) / 2$, based on the recorded absorbance at these wave lengths (following Yampolsky *et al.*, 2014) and standardized by dry mass.

SUPPLEMENTARY INFORMATION B: FIGURES AND TABLES

Figures

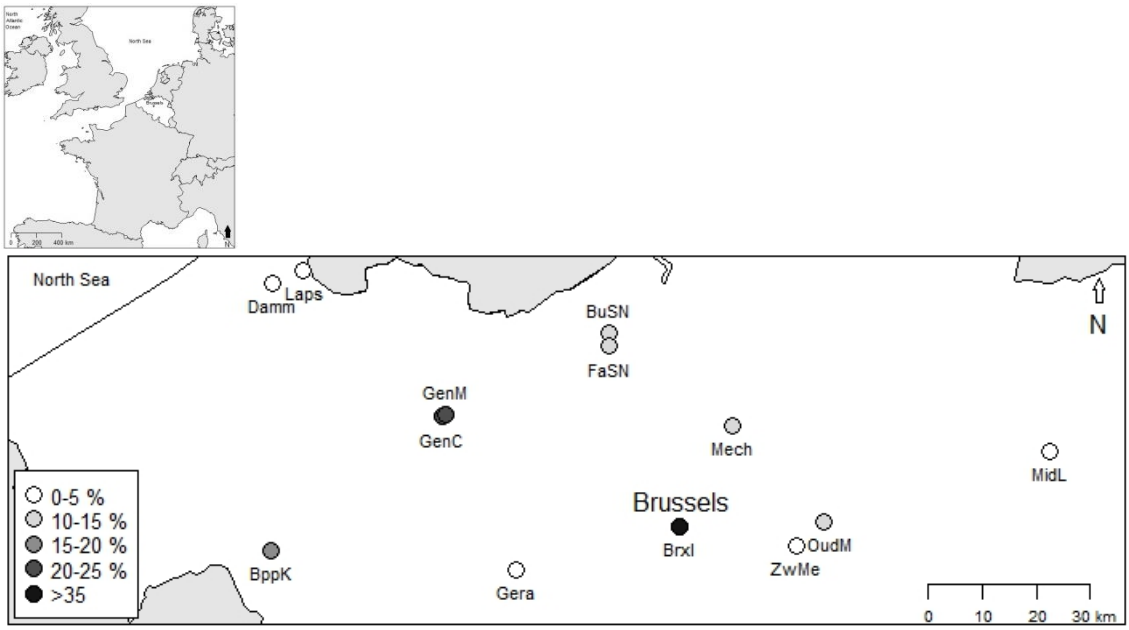


Figure S1. Sampling locations of the *D. magna* study populations in Flanders. Urbanization classes are given according to the percentage built-up area in a radius of 3200 m around each pond. Inset: Wider region of Europe, depicting Belgium.

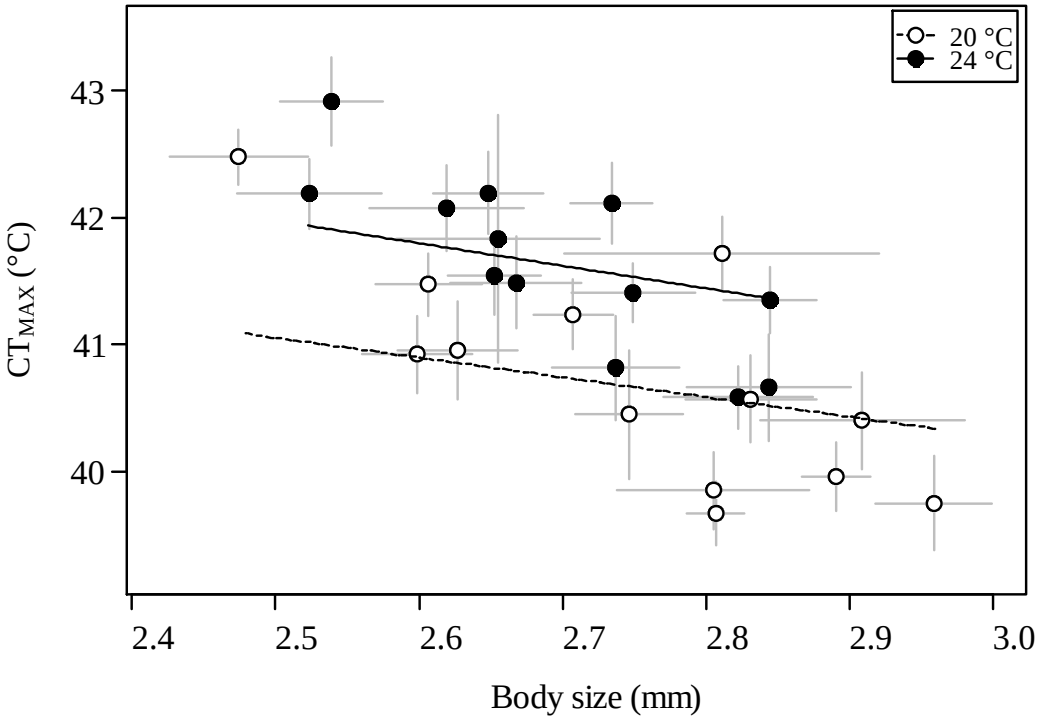
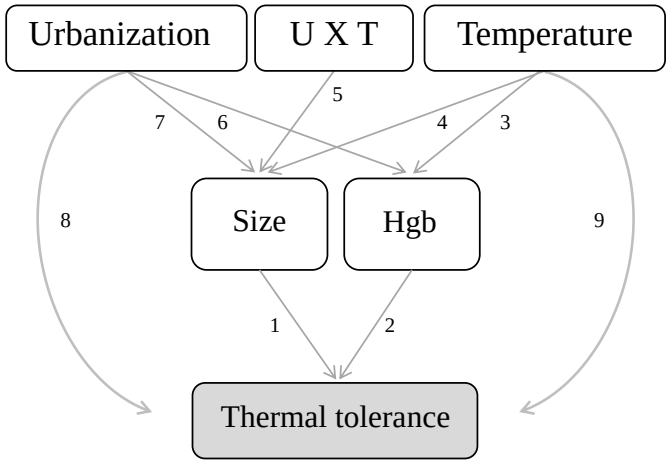


Figure S2. Critical thermal maxima of all 13 *Daphnia magna* populations plotted against body size (size at maturity). Population means are given with ± 1 SE. Regression lines are given for both rearing temperatures (20 °C: open symbols, dashed line; 24 °C: filled symbols, full line). based on the slope in the mixed model in which CT_{MAX} was regressed against body size (size at maturity).



Figures S3. Confirmatory path analysis diagram. *A priori* pathways were hypothesized between exogenous (T: temperature treatment, U: urbanization) and endogenous variables (size, haemoglobin concentration, and thermal tolerance) based on literature. (1) Geerts *et al.*, 2015; (2) Williams *et al.*, 2011; (3) Seidl *et al.*, 2005 (4) Atkinson, 1994; (5) Brans *et al.*, 2017a; (6) Harris *et al.*, 2013; (7) Brans *et al.*, 2017a, Magura *et al.*, 2006; (8) Angiletta *et al.*, 2007; (9); Yampolsky *et al.*, 2014.

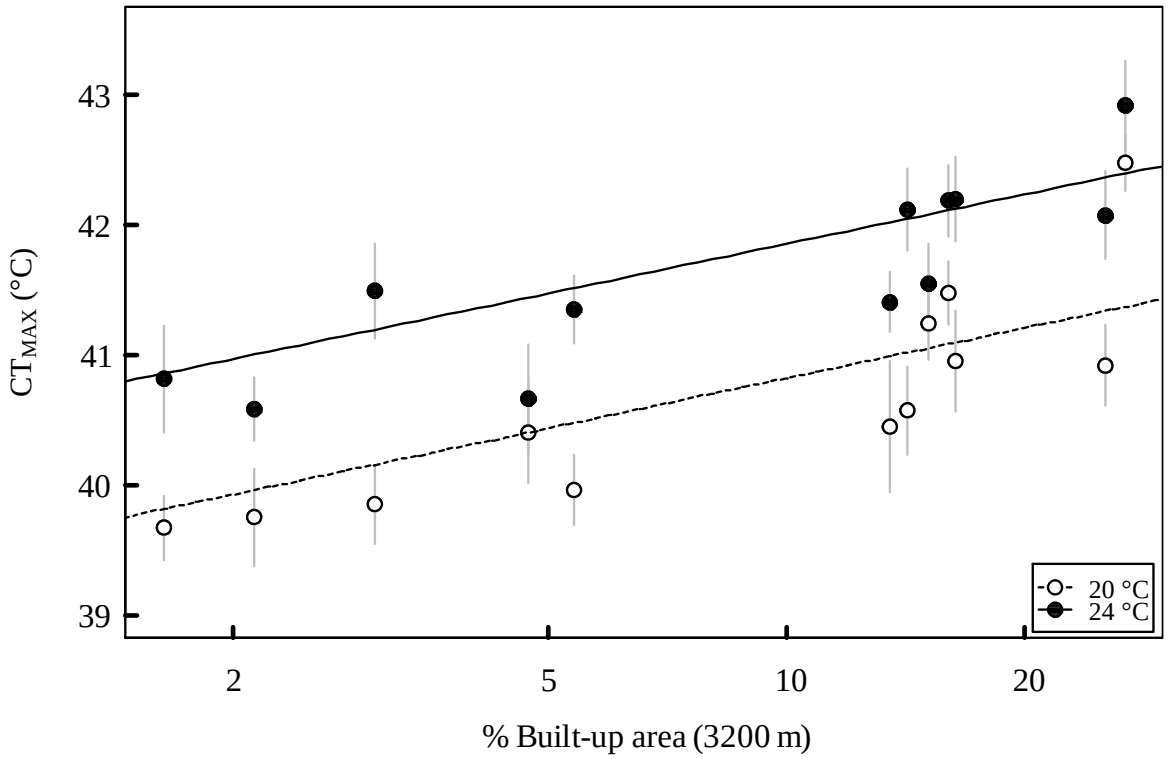


Figure S4. Average critical thermal maxima plotted against the level of urbanization for all *D. magna* populations, excluding the *Brxl* population. Given are population means ± 1 SE when reared at 20 °C (open symbols; dashed line) and at 24 °C (filled symbols; full line). The urbanization level is plotted as percentage built-up area (+ 1, log-scale) within a radius of 3200 meter around each sampling location. Regression lines are given for both rearing temperatures based on the slope in the mixed model.

Tables

Table S1. Locations of the *D. magna* study populations including the percentage built-up area (BA) in a radius of 3200 m around each pond and the number of experimental lines and multi-locus genotypes (MLGs) used in the experiment.

Population		**Location**		**BA (%)**	**Exp. lines**
Pond	Code	Latitude (N)	Longitude (E)	3200 m	(MLGs)
Brussel	*Brxl*	50.855464	4.347334	40.440	4(4)
Damme	*Damm*	51.26207	3.276039	1.126	6(6)
Gent (Citadelpark)	*GenC*	51.03890	3.723744	24.242	6(6)
Gent (Muinkpark)	*GenM*	51.04251	3.731611	25.736	6(5)
Geraardsbergen	*Gera*	50.78420	3.915978	4.382	6(3)
Kortrijk (Blauwe Poort)	*BppK*	50.81624	3.271563	15.356	6(6)
Lapscheure	*Laps*	51.28253	3.355467	0.637	6(5)
Leuven (Oude-Meren)	*OudM*	50.86328	4.723935	13.188	6(5)
Mechelen (Kruidtuin)	*Mech*	51.02402	4.484039	14.125	6(2)
Meerdaal (Zoete Waters)	*ZwMe*	50.82274	4.653691	2.017	6(6)
Midden-Limburg (BKN1)	*MidL*	50.98233	5.317858	3.721	6(5)
Sint-Niklaas (Bufferbekken)	*BuSN*	51.17808	4.161051	12.479	6(4)
Sint-Niklaas (Fabiola Park)	*FaSN*	51.15623	4.159557	14.995	6(5)

Table S2. Microsatellite markers (n = 27) used to genotype all field isolates, structured in four multiplexes (*MO1*, *MO2*, *MO3* and *MO5*). Cycling conditions for multiplex pairs *MO1* - *MO2* and *MO3* - *MO5* were identical. *EST4276* was an additional marker added to the *MO1* multiplex. Adapted from Jansen *et al.* (2011b) and Orsini *et al.* (2012).

	Multiplex			
	MO1	*MO2*	*MO3*	*MO5*
Markers	B050 B064 B074 B045 B030 B107 B008 B096 + *EST4276*	B031 B065 B088 B174 B075 B155 B135	B010 B164 B133 B150 A001	B087 A002 B033 B011 B052 B180
Cycling conditions	95 °C - 15'00" 94 °C - 00'30" 56 °C - 01'30" 72 °C - 01'30" 60 °C - 30'00"	95 °C - 15'00" 94 °C - 00'30" 56 °C - 01'30" 72 °C - 01'30" 60 °C - 30'00"	95 °C - 15'00" 94 °C - 00'30" 54 °C - 01'30" 72 °C - 01'30" 60 °C - 30'00"	95 °C - 15'00" 94 °C - 00'30" 54 °C - 01'30" 72 °C - 01'30" 60 °C - 30'00"
	4 °C - end	4 °C - end	4 °C - end	4 °C - end
	Cycli: 27	Cycli: 27	Cycli: 27	Cycli: 27

Table S3. Results of the linear mixed-effect models for CT_{MAX} excluding the *Brxl* population. (a) Results for the fixed effects, and (b) results for the random effects. Size at maturity was included as covariate in the CT_{MAX} analysis. Significant p-values ($p < 0.05$) are given in bold.

a) Fixed effects	CT_{MAX}			
	F	Df	Df_{res}	p
Urbanization level	27.319	1	12.04	<0.001
Temperature	76.527	1	325.40	<0.001
Size (at maturity)	5.246	1	327.01	0.023
Urbanization x Temperature	0.069	1	324.23	0.793
Urbanization x Size	0.219	1	282.84	0.640
Temperature x Size	0.094	1	362.71	0.759
Urbanization x Temperature x Size	0.793	1	328.49	0.373

b) Random effects	CT_{MAX}		
	χ^2	Df	p
Population	0.003	1	0.955
Clone	5.945	1	0.015
Day	65.202	1	<0.001

Chapter 3

City life on fast lanes: urbanization induces an evolutionary shift towards a faster life style in the water flea *Daphnia*

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Abstract

1. Multiple species show significant trait shifts in response to urbanization. Yet, the impact of anthropogenic warming linked to the urban heat island effect is remarkably understudied. Additionally the relative contributions of phenotypic plasticity and genetic change underlying trait shifts in response to urbanization are poorly known.
2. A common garden study with the water flea *Daphnia magna* revealed that both genetic differentiation in response to urbanization and phenotypic plasticity in response to higher rearing temperature (24 °C) induced significant parallel multivariate shifts in life-history strategy along the slow-fast pace-of-life axis.
3. Urban animals and animals reared at higher temperatures are characterized by fast maturation, early release of progeny, a smaller size at maturity, increased fecundity, and higher performance (given by maximal population growth rate *r*) compared to genotypes isolated from rural ponds and animals reared at lower temperatures respectively.
4. Evolution in response to urbanization accounted for 30% of the total observed shift in life history and caused a significant change in mean trait values, while plasticity responses to experimental warming were unaltered between urban and rural populations. The total trait change achieved through both plasticity and evolution ranged from 8 to 56% depending on the trait.
5. Our results provide clear evidence for evolution underlying an increase in pace-of-life of populations in response to urbanization. Given the pivotal role of *Daphnia* in aquatic ecosystems, this shift potentially feeds back to population structure, top-down control of algae, and food web dynamics in urban freshwater ecosystems. Additionally, we argue that adaptation to urban heat islands might render these populations pre-adapted in a context of future climate change.

INTRODUCTION

Urban environments are increasingly dominant in contemporary landscapes, and represent an exceptional combination of different dynamic abiotic and biotic disturbance gradients: cities are more chemically and physically polluted, eutrophied, fragmented, noisy, warmer, artificially lighted, and have a higher occurrence of exotic species compared to rural and natural areas (Gilbert, 1989; Kaye *et al.*, 2006; Grimm *et al.*, 2008a,b; Ramalho & Hobbs, 2012; Douglas & James, 2015; Knop *et al.*, 2017). These profound and complex environmental changes induce fast and pronounced ecological and evolutionary changes in many species (Cheptou *et al.*, 2008; Donihue & Lambert, 2015; Parris, 2016; Alberti *et al.*, 2017a,b; Brans *et al.*, 2017a,b). As a consequence of the earlier onset of growing seasons in urban areas, many plant species show changes in flowering time, size, and seed production (Neil & Wu, 2006; Palma *et al.*, 2016), while birds shift egg laying date and mating behavior (Slabbekoorn *et al.*, 2013; Cardoso, 2014; Møller *et al.*, 2015). Most studies, however, report on field observations, and thus confound genetic differences and phenotypic plasticity responses. It has been questioned whether phenotypic plasticity is sufficient to assure long-term population persistence of species when exposed to strong environmental disturbances (Fuller *et al.*, 2010; Duputié *et al.*, 2015; Gunderson & Stillman, 2015; Diamond & Martin, 2017). As more studies engage in common garden approaches, the evidence of genetic differentiation between rural and urban populations for physiological, morphological, life-history, and behavioral traits is rapidly growing (Cheptou *et al.*, 2008; San Martin Y Gomez & Van Dyck, 2012; Yakub & Tiffin, 2016; Brans *et al.*, 2017a,b). A substantial number of studies report clear signals of urban adaptations using percentage built-up area or impervious surfaces in the surroundings of the habitat, or distance from the city center. These metrics serve as a proxy for urbanization, integrating all environmental disturbances associated with city environments. Urbanization is a complex mixture of environmental disturbances and often cannot be traced back to one specific environmental axis. As not all abiotic and biotic changes associated with urbanization can be properly quantified (e.g. some pollution events are short-lived), the approach of using percentage built-up area or impervious surfaces as a proxy is a suitable and straightforward method to assess adaptations in response to the multitude of stressors organisms are exposed to when living in cities. For example, Sprau *et al.* (2016) found that the phenotypic variation in great tits (*Parus major*) inhabiting urban and rural plots, known to differ in temperature, humidity, and light conditions, were best explained by the simple dichotomy 'urban/rural' in comparison with using the specific environmental variables assessed in all plots.

A well-known extension to classical life-history theory (Roff, 1992, 2002; Stearns, 1992) is the 'pace-of-life' (POL) concept (Saether, 1988; Promislow & Harvey, 1990; Ricklefs, 2000; Wiersma *et al.*, 2007), in which individuals, populations, and species are ranked along a 'slow-to-fast' pace-of-life axis reflecting covariation among life-history traits (Réale *et al.*, 2010; Debecker *et al.*, 2016). 'Fast living' organisms are characterized by rapid development and maturation, small body size, high fecundity, and often reduced lifespan. 'Slow living' organisms fall on the opposite end of the continuum.

This concept is closely linked to the physiological processes that support it (i.e. the life-history/physiology-nexus; Ricklefs & Wikelski, 2002; Londoño *et al.*, 2015), and other organismal traits, together synthesized in the Pace-of-life Syndrome (POLS) hypothesis. This POLS hypothesis states that physiological, behavioral, and immunological characteristics of closely related species or populations experiencing contrasting environments covary with specific life-history traits evolved in these contrasting environments (Brodin & Johansson, 2004; Réale *et al.*, 2010; Debecker *et al.*, 2016; Monceau *et al.*, 2017; Segev *et al.*, 2017). The POLS hypothesis has been studied in a broad range of species, from invertebrates (Van Voorhies & Ward, 1999; Niemelä *et al.*, 2013; Debecker *et al.*, 2016) to birds (Wikelski *et al.*, 2003; Londoño *et al.*, 2015) and mammals (Oli, 2004; Nilsen *et al.*, 2009). Yet most studies on pace-of-life divergence focus on between-species level differences (e.g Debecker *et al.*, 2016.) or shifts between populations along large scale climatic gradients (e.g. across latitudes; Londoño *et al.*, 2015). Evidence on pace-of-life divergence along small scale climatic gradients is remarkably understudied.

Anthropogenic activity, energy consumption, and the radiation of heat by buildings at night cause cities worldwide to heat up, a general and, more important, re-occurring phenomenon known as the 'urban heat island effect' (Oke, 1973, 1982; Santamouris, 2007; Ward *et al.*, 2016). For aquatic ecosystems such as freshwater ponds and pools, a similar urban heat island effect (i.e. an urban 'hot-tub effect') was recently reported, with city ponds having higher mean, minimum, and maximum temperatures (Brans *et al.*, 2018a; see also Fig. S1, Supplementary Information). In this study, it was found that the average summer mean and maximum temperature differences between city and rural ponds reached 3.04 °C and 3.69 °C, respectively. We recently reported a genetic increase in heat tolerance in urban compared to rural populations of the water flea *Daphnia magna* (Brans *et al.*, 2017b). Such an increase in heat tolerance was also reported in a study on urban thermal adaptation in a terrestrial organism, acorn ants (Diamond *et al.*, 2017b). Yet, warming is also intrinsically related to key life-history traits such as growth rate, fecundity, and survival (Angilletta, 2009). In addition, the urbanization gradient might also include other stressors (e.g. increased levels of disturbance such as those linked to the management of the water bodies) that might select for differences in life histories. Although recent studies explored phenotypic life-history shifts in city dwellers (Sprau *et al.*, 2016; Charmantier *et al.*, 2017; Sol *et al.*, 2018), surprisingly little is known on genetic differences underlying pace-of-life in response to urbanization. Especially ectotherm animals, whose metabolic rates and growth are substantially driven by environmental temperatures, will be prone to respond to higher average and maximum temperatures, and increased temperature fluctuations (Angilletta *et al.*, 2004; Deutsch *et al.*, 2008; Daufresne *et al.*, 2009; Hoffmann *et al.*, 2013). It is not straightforward to predict the direction of evolutionary responses to urban heat islands and other urbanization-linked stressors. In ectotherm species, one might predict that warming in cities might induce evolution of a fast life cycle involving small individuals that develop fast and produce many, smaller eggs. This prediction is in line with the

temperature-size rule (Atkinson, 1994; Forster *et al.*, 2012; Hoefnagel & Verberk, 2015) and complements the earlier observation that small organisms have a higher thermal tolerance, as was also reported for *Daphnia* (Geerts *et al.*, 2015; Brans *et al.*, 2017b). The occurrence of disturbances linked to management (e.g. clearance of litter layer on the bottom of the pond and addition of water; Hassall, 2014; personal observations) and extreme temperature events in cities (Ward *et al.*, 2016; Wouters *et al.*, 2017; Brans *et al.*, 2018a) might also select for a fast life cycle (Evans *et al.*, 2010; Atwell *et al.*, 2012; Charmantier *et al.*, 2017). Alternatively, the limited availability of oxygen in aquatic systems and the increased oxygen demand at higher temperatures (Bozinovic & Pörtner, 2015; Chown *et al.*, 2015; Horne *et al.*, 2015) might lead to the expectation of lower metabolic rates and thus a slower life style in urban compared to rural populations of ectotherms (Angilletta *et al.*, 2004; Tüzün *et al.*, 2017b). One might also expect countergradient responses, such as those recently shown by Tüzün *et al.* (2017b) in damselflies, where urban populations genetically grow slower (at all tested rearing temperatures in the laboratory) compared to rural populations as a likely response to longer growing seasons in urban ponds (Brans *et al.*, 2018a), that result in relaxed time constraints for urban animals.

Here we test whether urban populations of *Daphnia magna*, a key grazer in the pelagic of shallow, eutrophic ponds and lakes, are genetically differentiated from rural populations for their life-history traits, and whether these changes are situated along the slow-fast life-history axis, in line with the pace-of-life hypothesis (Réale *et al.*, 2010). We performed a common garden experiment using *D. magna* genotypes originating from a total of 13 populations along strong urbanization gradients. To quantify the relative importance of evolution of mean trait values, plasticity to warming, and evolution of thermal plasticity, animals were reared at two temperatures (20 °C and 24 °C), approximating the temperature differences currently observed between rural and urban ponds in Flanders (Northern Belgium) during spring and summer (Brans *et al.*, 2017a; Brans *et al.*, 2018a; and see Fig. S1 Supplementary Information,). We quantified a set of eight life-history traits and assessed genetic differentiation (rural vs. urban), plastic responses for the range 20- 24 °C, and interactions between evolution and plasticity across this temperature range (urbanization-dependent thermal plasticity). We address trait divergence from both a univariate and multivariate approach, testing the hypothesis that urban populations have genetically adapted to live 'faster', i.e. have a higher growth rate, mature earlier, and release their young faster. Although a fast life-history strategy might translate in the production of more offspring, rapid development and maturation at a smaller size potentially comes at a cost of the production of smaller clutches. In summary, this study is the first to tap in to evolutionary pace-of-life divergence along urbanization gradients and additionally aims to quantify the relative contribution of evolution, phenotypic plasticity, and evolution of plasticity to the total observed intraspecific phenotypic trait changes observed between rural and urban populations. Our common garden approach does go beyond showing phenotypic responses, as it allows disentangling the importance of phenotypic plasticity and evolutionary divergence to the observed phenotypic trait shifts in response to urbanization.

MATERIAL AND METHODS

Study organism and urbanization gradient

The populations and genotypes used in the present study are identical to those in our earlier study reporting evolution of increased thermal tolerance (Brans *et al.*, 2017b). Thirteen populations of the water flea *D. magna* were sampled in Flanders (Northern Belgium) in the summer of 2013, and categorized according to the surrounding percentage built-up area (Fig. 1, 3200 m radius, reflecting regional levels of urbanization; see Fig. S2, Supplementary Information, for correlations with measurements of %BA at smaller spatial scales) into urban (> 10%) and rural (< 5%). %BA refers to the percentage surface covered by buildings and does not include roads and parking lots. As a result, areas characterized by more than 10% of BA are already substantially urbanized (see Fig. 1). Rural populations were characterized by an additional criterion of minimum 20% of Biologically Valuable Area in the regional surroundings of the pond (3200 m radius). Detailed information on sampling locations, the assessment of urbanization, and pre-experimental culturing procedures can be found in Supplementary Information (Supplementary Information A; Supplementary Information B).

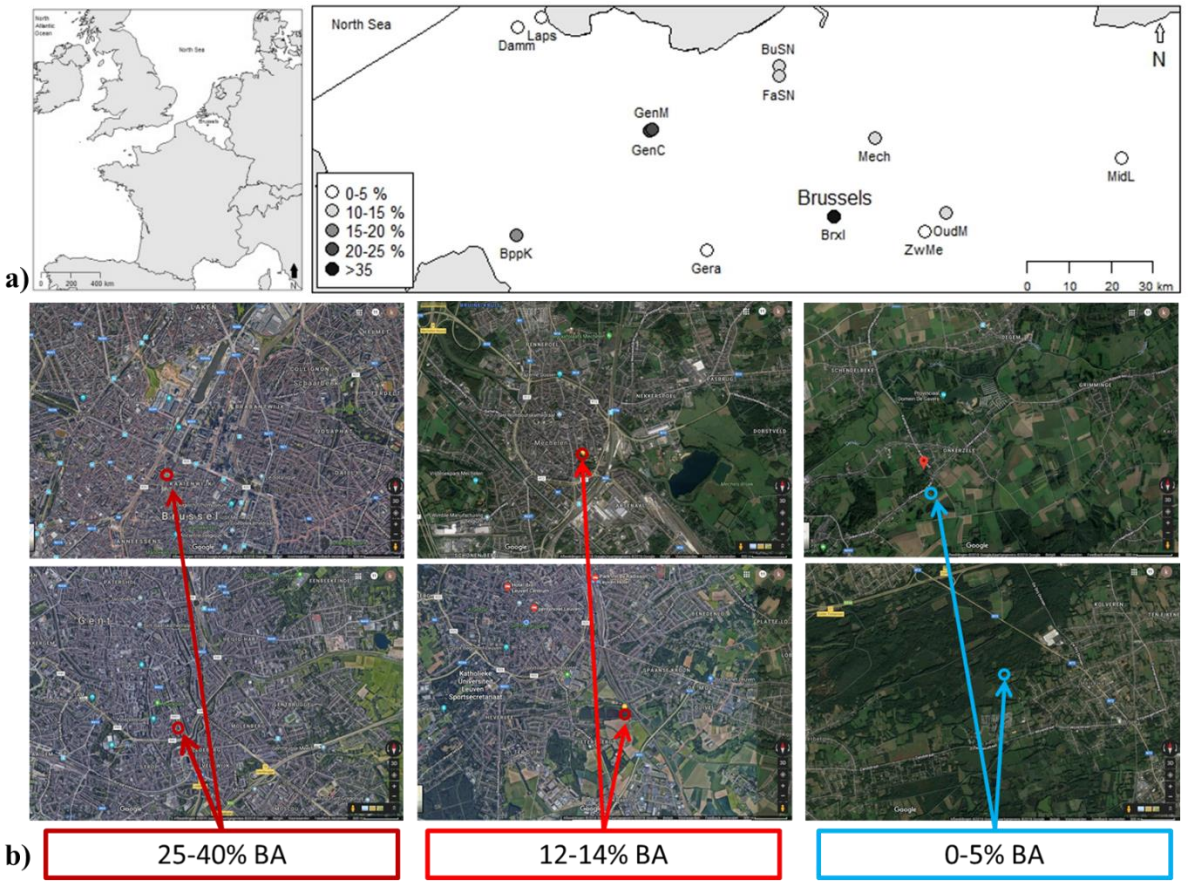

Figure 1. (a) Sampling locations of the *D. magna* study populations in Flanders (Inset: Wider region of Europe, depicting Belgium). Urbanization classes are given according to the percentage built-up area in a radius of 3200 m around each pond. Ponds surrounded with more than 10% of built-up area are considered urban. Rural ponds have an additional criterion of 20% Biologically Valuable area at a radius of 3200 m around the pond. (b) Examples of sampled ponds in urban settings (25-40% BA: upper: city of Brussels; lower: city of Ghent; 12-14%: upper: city of Mechelen; lower: city of Leuven) and in rural settings (category 0-5% BA) to illustrate the point that >10% BA represents highly urbanized settings. The circles represent the sampling locations (ponds). All ponds are located here in a plot of ca. 6 x 4 km here, in which the regional scale of urbanization is in concordance with the 3200 m radius at which %BA was assessed

Experimental set-up and response variables

Overall design and response variables

To test for an effect of urbanization level and temperature treatment on *D. magna* life-history traits, we carried out a full factorial experiment (design: 13 populations x 6 independent lines x 2 temperatures; 20 °C and 24 °C; *Brxl* only 4 lines; each combination in triplicate except *Brxl*: one replicate; see also Supplementary Information A) resulting in 440 experimental units. After purging maternal and grandmaternal effects (See Supplementary Information A), a cohort of 12 juveniles (< 24h old) of each culture was inoculated in a 500ml jar and assigned to the appropriate temperature treatment (water baths set at 20 °C and 24 °C, 14:10 L:D photoperiod). Medium refreshment and feeding were standardized (see Supplementary Information A). We measured size at birth for three individuals for each experimental cohort. At maturity, six animals per experimental cohort were followed-up to quantify life-history traits (the six others were scored for thermal tolerance; see Brans *et al*., 2017b).

Life-history traits

The experimental cohorts (n = 220 per temperature treatment) were monitored on a daily basis and the following life-history traits were scored: age and size at maturity, age at release of the first and second clutch, and the number of juveniles in the first and second clutch (number of juveniles/female, to assess fecundity). Size measurements were performed on four animals per cohort (measured from the center of the eye to the base of the tail spine). From these life-history measures we estimated two additional life-history traits: somatic growth *Gj*, calculated as *Gj = (ln (size at maturity) – ln (size at birth))/age at maturity*, and individual performance *r*, calculated by iteratively solving the Euler equation to 1 ($\sum e^{-rx} l_x m_x = 1$; Stearns, 1992). This latter measure of performance translates into population growth rate assuming no variation between individuals.

Statistical analysis

All statistical analyses were performed with the R software version 3.3.2 (R Development Core Team, 2016).

MANOVA and univariate mixed-effect models

To test for overall differentiation in life-history traits between urban and rural *Daphnia*, and between animals reared at 20 °C versus 24 °C, we performed MANOVA on all response variables scaled to a mean of 0 and standard deviation of 1. To test for differentiation in the different life-history traits between urban and rural *Daphnia*, and between animals reared at 20 °C and 24 °C, we subsequently ran separate linear mixed-effect models for each of the eight response traits. For both the MANOVA and the univariate mixed-effect models, we used urbanization, temperature treatment and their interaction term as fixed factors, and population nested in urbanization, and clone nested in population, as random

effects. To assess a possible effect of clonal identity (implying genetic variation for a certain trait), we applied model comparison of linear mixed-effect models with and without this random factor. The computation of test statistics, the multivariate and univariate model specifications, assumption verifications, and used R packages are detailed in Supplementary Information A. We additionally tested random slopes models for each linear mixed-effect model to control for any clone or population specific G x E interactions (E: temperature treatment), thus allowing both intercepts and slopes to vary between clones and populations (random factors) within urbanization-level in response to temperature. The results of these models were qualitatively identical to the random intercept models, and AIC score comparisons only indicated a higher preference for the random slopes model in two (age at maturity, release of second clutch) out of eight measured variables. In both cases however, results were the same (for release of the second clutch the effect of urbanization changed from marginally significant to significant – $p = 0.014$, see Table 1 of the Results for comparison). We here thus overall report random intercept models.

Principal Component Analysis (PCA) and Phenotypic Trajectory Analysis (PTA)

To complement MANOVA results and visualize the multivariate responses, we conducted PCA (Principal Component Analysis) and PTA (Phenotypic Trajectory Analysis; Collyer & Adams, 2007; Adams & Collyer, 2009) to: (i) detect the principal modes of variation and covariation among the measured life-history traits, (ii) better describe and visualize the multivariate phenotypic trajectories of the populations for the studied temperature range (20-24 °C), (iii) test for multivariate convergence or divergence of urban and rural populations within temperature treatments, and (iv) test for the evolution of multivariate reaction norms (Plaistow & Collin, 2014). PTA was performed using the approach described in Collyer & Adams (2007) and Stoks *et al.* (2016), applying a residual randomization procedure, and including random effects and nested designs (population, nested in urbanization, and clone, nested in population, both as random factors). In short, this method first allows calculating the length (magnitude) and angle (or direction, θ) of multivariate vectors of trait change (here: between temperature treatments) and enables to compare these between groups (Fig. 2a dashed lines). The method can further be extended to test for multivariate convergence or divergence of populations by comparing the orientation of the reaction norms (Dennis *et al.*, 2011) and the Euclidean distances between centroids (Fig. 2b full lines). This approach thus can also be used to assess whether groups are distinct in multivariate trait space, which in the current case tests whether the difference in the trajectory of the multivariate genotypic trait values between urban and rural populations in a certain temperature treatment is significantly different from 0 (see 'evolutionary trajectories' Fig. 2b). PTA and PCA procedures are described in detail in Supplementary Information A.

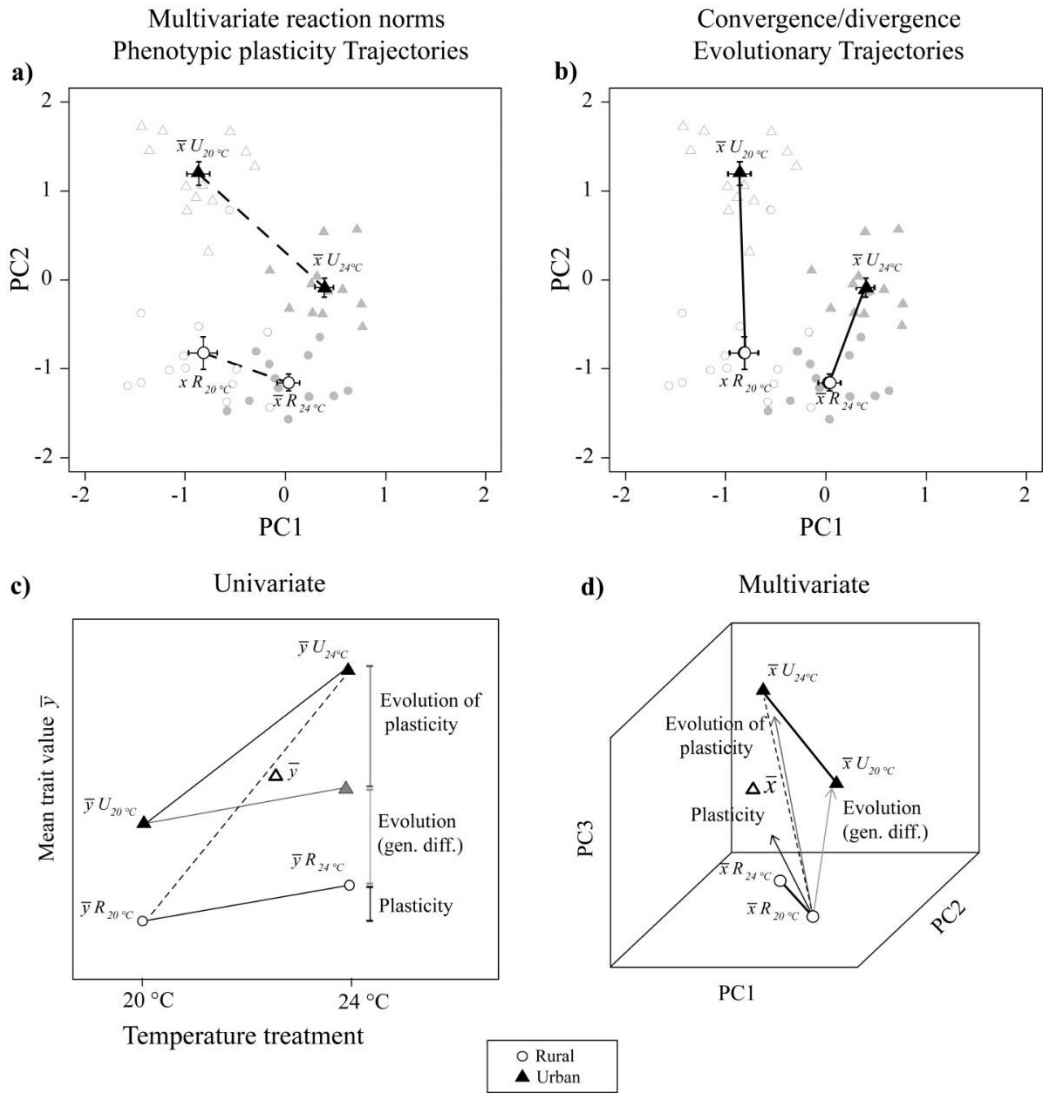

Figure 2. Schematic methodological illustrations. Upper plots: Principal Components Analysis plots schematically illustrating how Phenotypic Trajectory Analysis (PTA) was applied to (a) test for differences in the magnitude and angle of multivariate reaction norms (i.e. 'phenotypic plasticity trajectories') of, for instance, rural (circles) and urban (triangles) populations (small grey symbols here indicate population means, large black symbols are group means), reared at 20 °C (open grey symbols) and 24 °C (filled grey symbols), and (b) test for convergence or divergence and genotypic differentiation (referred to as 'evolutionary trajectories'; symbols are colored likewise); adapted from Stoks *et al.* (2016). Lower plots: Illustrative example adapted from Govaert *et al.* (2016) of (c) the univariate reaction norm for a certain phenotypic trait Y, with mean trait values for urban (U, black triangles) and rural (R, white circles) *Daphnia* reared at 20 °C and 24 °C (indicated in index), and (d) the multivariate reaction norm for a set of *n* phenotypic traits. Under the assumption that evolution occurs from rural to urban populations, total observed trait change ($\Delta\bar{y}$, dashed line) can be partitioned into three components, as in Govaert *et al.* (2016): evolution (genotypic differentiation between rural and urban *Daphnia*), plasticity (here thermal plasticity given by the difference in trait values between 20 °C and 24 °C), and evolution of plasticity (difference in reaction norm slopes of rural and urban *Daphnia*). To visualize multivariate reaction norms in 3D-space, we first perform a Principal Component Analysis on the set of *n* phenotypic traits. The position of the group averages is then plotted for urban and rural populations at 20 and 24 °C along the first three PC axes; given that the amount of variation explained by the three PC axes rises, the Euclidian length of $\Delta\bar{x}$ represents the best approximation of the true length of $\Delta\bar{y}$.

The relative contribution of univariate and multivariate evolution, phenotypic plasticity, and the evolution of plasticity to total trait change

We quantified the relative contribution of both multivariate and univariate plastic (phenotypic plasticity to warming) and genetic (evolution of mean trait values and evolution of plasticity) changes to the total phenotypic trait change between rural and urban populations of *D. magna* under the prevailing temperature conditions in the field in the respective habitats (i.e. for the studied temperature range 20-24 °C). We applied the reaction norm approach described in Govaert *et al.* (2016) and Stoks *et al.* (2016) (see Fig. 2c,d). To this end, we assume that the rural populations are the 'ancestral' state (note they are characterized by a min. of 20% Biologically Valuable Area and thus reflect pristine Flemish habitats most closely) and that trait change between populations occurred during the transition of populations inhabiting colder, rural habitats to populations inhabiting warmer, urban habitats. We assume an average summer temperature of rural ponds of 20 °C and an average temperature increase in urban ponds of 4 °C, which broadly corresponds to the average summer temperature in rural ponds and the urban heat island effect measured in urban ponds (Brans *et al.* 2018a: difference in daily mean temperature between urban and rural ponds: ΔT_{mean} : +3.04 °C; difference in daily maximum temperature ΔT_{max}: +3.69 °C; data on a more limited set of ponds reported by Brans *et al.* 2017a indicate ΔT_{mean} : +3.93 °C and T_{max}: +4.03 °C; see also Fig. S1 in this Supplementary Information).

We additionally estimated 'evolutionary gain' for every trait, which quantifies the observed evolution-driven trait change achieved by urban compared to rural populations when exposed to warming. Evolutionary gain was calculated as the difference between the urban and rural mean trait values for each trait when reared at 24 °C, divided by the trait value of rural animals at 24 °C, and thus reflects the percentage of trait change that was additionally achieved through evolution when exposed to the warmer environment.

RESULTS

Multivariate MANOVA and univariate linear mixed-effect models

MANOVA results indicated overall significant differences in life-history traits between rural and urban genotypes ($p < 0.001$; Table 1) and a significant plasticity response in these traits in response to rearing temperature ($p = 0.046$; Table 1). The associated univariate linear mixed-effect models showed that urban animals mature at a smaller size ($p = 0.003$, Fig. 3a) and earlier ($p = 0.019$, Fig. 3b), were younger at early reproduction (release first clutch: $p = 0.043$, release second clutch: marginally significant, $p = 0.050$) and had a higher fecundity (number of juveniles in second clutch: $p = 0.040$; first clutch: not significant, $p = 0.069$) (Fig. 3c-f). Somatic growth did not significantly differ between urban and rural genotypes ($p = 0.386$, Fig. 3g), whereas performance (intrinsic population growth rate 'r') tended to be higher in the urban genotypes (marginally significant, $p = 0.051$, Fig. 3h). While for size at maturity a significant interaction effect (urbanization x temperature treatment) was found ($p = 0.036$, Table 1, Fig. 2a), MANOVA results indicate no overall effect of urbanization x temperature treatment on the combined set of response variables, thus indicating multivariate plasticity responses to temperature are similar for the urban and rural genotype sets. Phenotypic plasticity responses in response to increased rearing temperature for all traits were in the same direction as responses to urbanization: smaller size at maturity, earlier age at maturity and at release of clutches, higher number of eggs in the first clutch, higher somatic growth, and higher individual performance ('r') (Table 1, Fig. 3). Only number of juveniles in the second clutch did not significantly differ between animals reared at 20 and at 24 °C ($p = 0.123$; Table 1).

Model comparison of linear mixed-effect models with and without the random effect of clone within the rural and urban clone sets indicated significant effects of this factor for three out of eight variables (Table 1): individual performance ($p = 0.01$), age at maturity ($p = 0.006$), and size at maturity ($p < 0.001$). This indicates significant clonal variation [for these three traits] within the urban and rural clone sets for these traits.

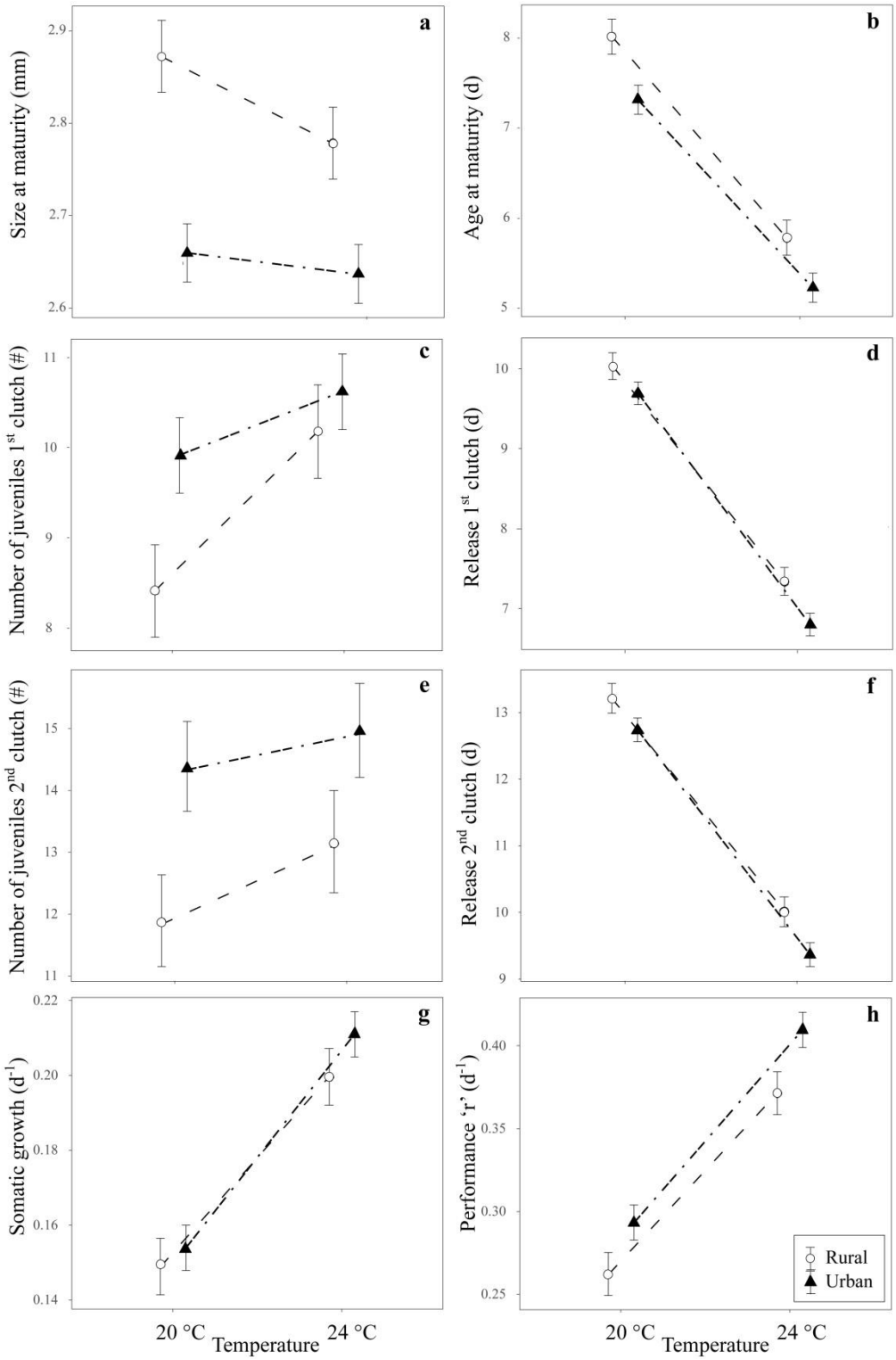

Figure 3. Univariate results. Mean (± 1 SE) size at maturity (a), age at maturity (b), number of juveniles in and the timing of release of the first (c-d) and second (e-f) clutch, somatic growth (g), and individual performance (h), for urban (black triangles) and rural (white circles) *Daphnia*, as a function of rearing temperature (20 °C, 24 °C; reaction norms: dashed lines; n = 220 per temperature treatment). Means and standard errors are obtained via model outputs of the linear mixed-effect models, accounting for random and nested data structure.

Multivariate trait space and trajectory analysis

PTA and convergence/divergence analysis reveals no significant difference in the size and angle of the multivariate phenotypic plasticity trajectories between urban and rural *D. magna* genotype sets (with both genotype sets being represented by multiple populations and genotypes nested in populations) when reared at 24 °C versus 20 °C (Fig. 4a,b, dashed lines, size difference = 0.304: p = 0.179; angle θ = 8.970°: p = 0.138). There is also no significant difference in size and angle of the evolutionary trajectories comparing rural and urban clone sets at each temperature (Fig. 4a,b, full lines, size difference = 0.031, p = 0.845; angle θ = 26°, p = 0.746). However, we find a clear difference in position in multivariate trait space between urban and rural populations (i.e. Euclidian distance between urban and rural centroids significantly different from 0; $p < 0.001$ at both temperatures), confirming earlier MANOVA results. The first three PC axes cumulatively explain 81.6% of multivariate trait variation and are strongly correlated with development time (age at maturity, at release of first, and at release of second clutch), somatic growth and performance (PC1, 49.2%), fecundity (PC2, 20.7%), and size at maturity (PC3, 11.7%). Combining the results of PTA and PCA indicates that both phenotypic plasticity in response to rearing at higher temperature (in rural as well as in urban populations) and genotypic differentiation (evolutionary trajectories) between rural and urban populations results in a shift towards a faster life style, with shorter development time, higher potential intrinsic rate of population growth, higher fecundity, and smaller body size.

Contributions of evolution, plasticity, and the evolution of plasticity to total trait change

We found a high relative contribution of evolution (i.e. evolution of a mean trait value combined with evolution of plasticity) to total observed trait change between rural and urban populations, assuming a shift from 20 to 24 °C, for traits: size at maturity (75.11%), number of juveniles in the first (59.18%) and number of juveniles in the second clutch (71.21%; Fig. 5a,b). Although lower, the relative contribution of evolution still accounts for ca. 25% of total trait change in two traits linked to pace-of-life: age at maturity (27.60%) and individual performance (25.96%). Within the component of evolutionary change, evolution of mean trait value contributes for all traits more to overall trait change (range 10.47% - 56.30%) than evolution of phenotypic plasticity, except for somatic growth (8.05% compared to 10.40%). Relative contributions of plasticity to the total observed phenotypic trait change (i.e. from rural populations reared at 20 °C to urban populations reared at 24 °C) is highest for release of first and second clutch (for both traits 83.25%) and somatic growth (81.57%). For multivariate trait change, evolution contributes 28.16% to total observed change in multivariate trait space (Fig. 5b). Evolutionary gain (Fig. 5c; Table S2, Supplementary Information B) is highest for performance (an increase of 10.30%), age at maturity (a reduction in time of 9.56%), and release of the first clutch (a reduction in time of 7.37%).

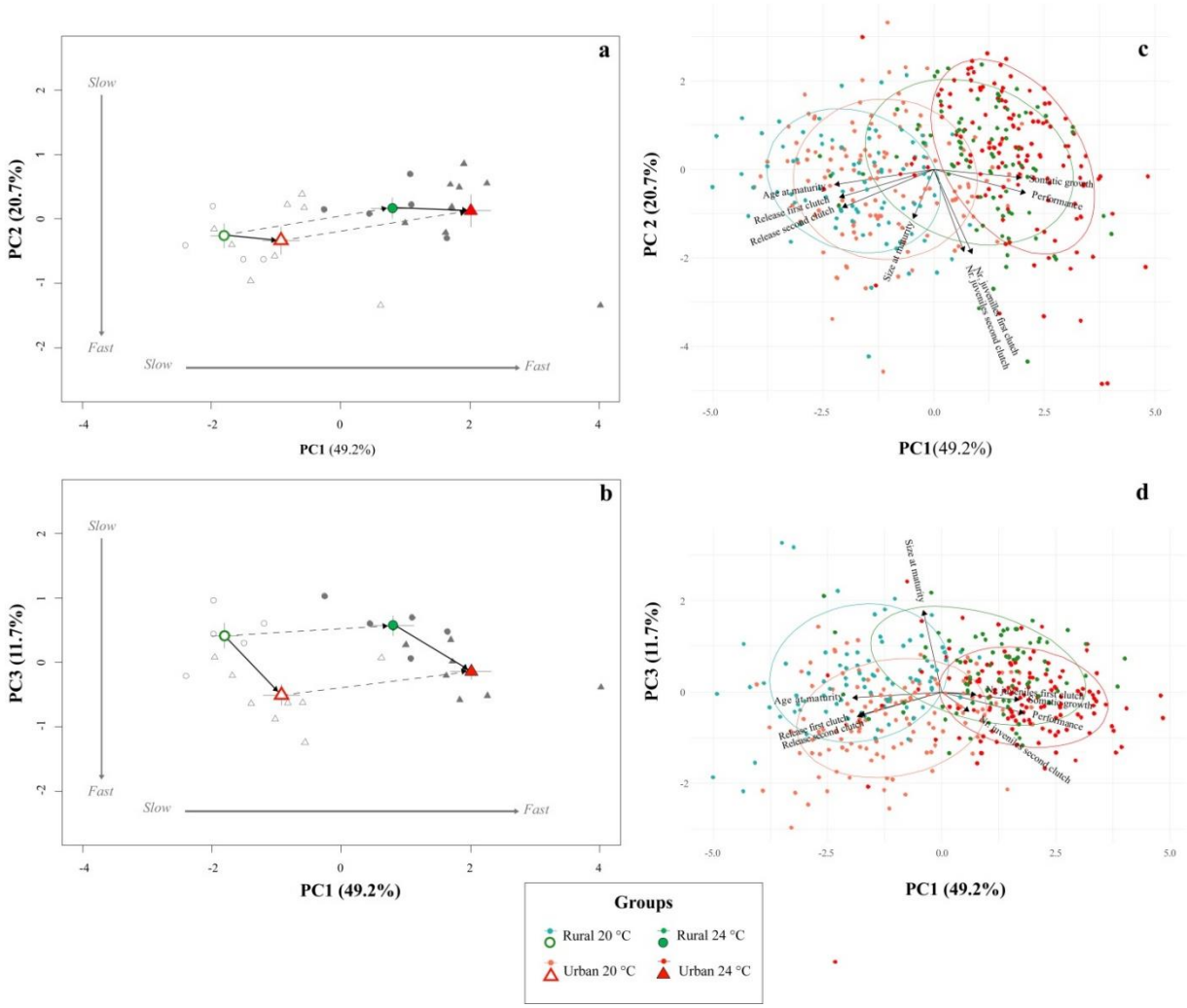

Figure 4. Multivariate results. Principal Component Analysis plots showing: (a,b) phenotypic plasticity trajectories (multivariate response to experimental warming for eight morphological and life-history traits; dashed lines) and evolutionary trajectories (multivariate genetic differentiation between rural and urban animals; full lines) for rural (green circles) and urban (red triangles) populations of the water flea *Daphnia magna* reared at 20 (open symbols) and 24 °C (full symbols); population means are depicted in grey shades (white: 20 °C, gray: 24 °C), average 'urban' and 'rural' centroids are depicted in the according color (rural: green; urban: red); (c,d) PCA plots showing all observations (n = 440) grouped according to the urbanization x temperature combinations (rural-20 °C: blue, rural-24 °C: green, urban-20 °C: orange, urban-24 °C, red). Patterns are given for PC1 and PC2 (a,c) and PC1 and PC3 (b,d). An indicative 'pace-of-life' axis along all PC axes is given in plots a and b.

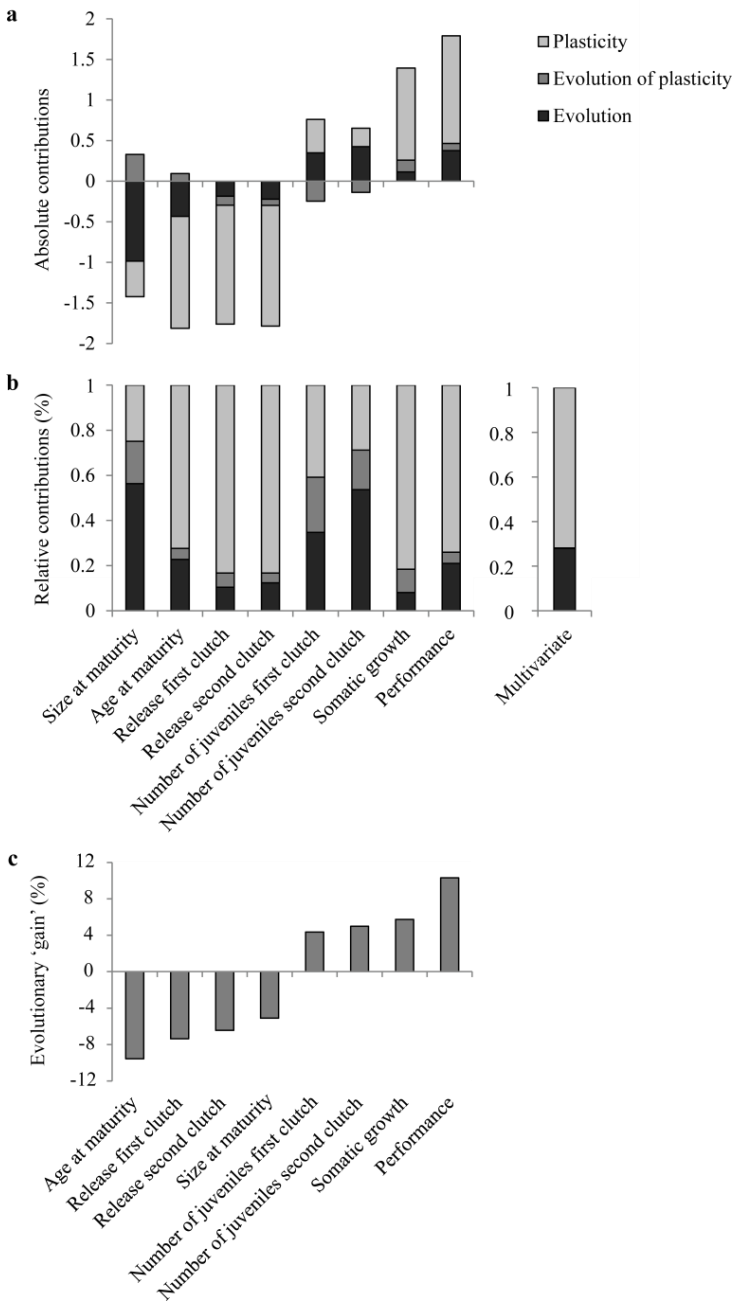

Figure 5. Eco-evolutionary variation partitioning. (a) Absolute and (b) relative contributions of evolution (black), evolution of (thermal) plasticity (dark grey), and phenotypic plasticity (light grey) to the standardized phenotypic change measured in eight life hisotory traits in urban and rural populations of *Daphnia magna*, reared at 20 and 24 °C (total nr. of observations: n = 440). Contributions are calculated according to Stoks *et al.* (2016) using the additive method. (c). Evolutionary gain, calculated for eight life-history traits in urban and rural *Daphnia magna* populations. Evolutionary gain is given as the percentage and direction of trait change for each trait (non-standardized, unit specific measure) that is realized due to evolution at the high temperature (i.e. comparing trait values of rural populations at 24 °C with trait values of urban populations at 24 °C). Evolutionary gain is ranked according to direction and percentage in the plot.

Table 1. Statistical results. Results of the MANOVA and univariate linear mixed-effect models, testing for the effect of urbanization level, treatment, and their interaction on eight morphological and life-history traits measured in *Daphnia magna*. Significant results ($p < 0.05$) are given in bold . Marginally significant results ($p = 0.05$) are marked with a '·'.

	Urbanization				**Temperature**				**Urbanization x Temperature**				**Clone**	
MANOVA	**F**	**Df**	**Pillai**	**p**	**F**	**Df**	**Pillai**	**p**	**F**	**Df**	**Pillai**	**p**		
All traits	5.119	8	**0.455**	**< 0.001**	2.171	8	0.262	**0.046**	1.453	8	0.192	0.199	*Included as error term*	
Univariate linear models	**F**	**Df**	**Df_{res}**	**p**	**F**	**Df**	**Df_{res}**	**p**	**F**	**Df**	**Df_{res}**	**p**	**χ^2**	**p**
Size at maturity	13.893	1	11.153	**0.003**	9.495	1	361.096	**0.002**	4.451	1	361.152	**0.036**	5.376	**< 0.001**
Age at maturity	7.525	1	10.845	**0.019**	403.273	1	364.024	**< 0.001**	0.468	1	364.183	0.494	7.534	**0.006**
Release 1st clutch	5.241	1	10.656	**0.043**	664.724	1	366.870	**< 0.001**	0.819	1	366.973	0.366	0.697	0.404
Number of juveniles 1st clutch	3.443	1	10.820	0.069	7.845	1	364.920	**0.005**	1.674	1	365.060	0.197	0.106	0.745
Release 2nd clutch	4.843	1	10.690	**0.050·**	655.013	1	366.61	**< 0.001**	0.402	1	366.730	0.526	0.004	0.998
Number of juveniles 2nd clutch	5.446	1	10.592	**0.040**	2.384	1	362.141	0.123	0.537	1	362.345	0.464	3.005	0.083
Somatic growth	0.816	1	10.840	0.386	281.354	1	366.162	**< 0.001**	0.932	1	366.316	0.335	0.272	0.602
Performance	4.777	1	11.139	**0.051·**	476.707	1	362.030	**< 0.001**	0.463	1	362.120	0.496	6.655	**0.010**

DISCUSSION

Cities generate strong and fast abiotic and biotic environmental disturbance gradients (Seto *et al.*, 2012; Parris, 2016). So far, urban evolutionary studies on aquatic ectotherms are extremely limited (Brans *et al.*, 2017a,b; Tüzün *et al.*, 2017b). Due to the urban heat island effect, water temperatures in small, standing waterbodies such as ponds and shallow lakes are considerably higher in urban than rural settings ($\Delta T_{mean\text{-}summer}$ = 3.04 °C; $\Delta T_{max\text{-}summer}$ = 3.69 °C; Brans *et al.*, 2018a, Brans *et al.*, 2017a), and this likely induces both thermal stress and oxygen limitation (Pörtner, 2001; Verberk *et al.*, 2016). Our results show that urban populations of the water flea *Daphnia magna* evolved a 'fast' life-history strategy and that both rural and urban populations exhibit a strong, nearly identical, plastic response to the applied change in rearing temperature (20 °C vs. 24 °C) for all measured traits. The direction of change, towards a faster pace-of-life, is very similar for the plasticity responses when exposed to higher temperature and for the genetic trait shift as one moves from rural to urban populations, for each of the individual life-history traits as well as for multivariate trait trajectories. Given the strong, intrinsic association between temperature and life-history traits in ectotherms, our results suggest that anthropogenic warming in cities shapes evolutionary pace-of-life strategies. Other stressors linked to urban environments, such as increased exposure to disturbances (such as heatwaves and artificial management; Hassall, 2014; Wouters *et al.*, 2017; Brans *et al.*, 2018a) might also have contributed to the observed genetic differentiation in pace-of-life in our study system. We discuss our results in more detail in the next paragraphs.

We found pronounced genetic differentiation in multiple life-history traits when considered individually (Table 1, Fig. 2) as well as in a multi-trait perspective (MANOVA, evolutionary trait trajectories Fig. 3 and Fig. 4). These genetic changes were along the slow-to-fast axis, with urban *Daphnia* populations being shifted towards the fast end of the 'pace-of-life' continuum. Urbanization causes a genetic shift towards faster maturation and faster release of early clutches, higher early fecundity, higher potential population growth rate, and smaller size at maturity. This response is, given the observed anthropogenic warming in the region (De Ridder *et al.*, 2015; Wouters *et al.*, 2017; Brans *et al.*, 2018a), in agreement with expectations of life-history adaptation to warming (cf. temperature-size rule; Atkinson, 1994; Angilletta, 2009). While the small body sizes of urban genotypes might be due to selection for short developmental time, it is most likely also due to or reinforced by the fact that smaller animals have a higher thermal tolerance (Geerts *et al.*, 2015; Brans *et al.*, 2017b). Although rapid maturation and small body sizes often come at a fecundity cost (Clarke, 2003), urban genotypes had a higher fecundity. This suggests selection for higher population growth rates (intrinsic population growth rate *r*). The observed genetic trait changes as one moves from rural to urban populations are indeed similar to those expected under "r"-selection (r-K selection; MacArthur & Wilson, 1967; Pianka, 1972; Stearns, 1976; Reznick *et al.*, 2002). While our observation of a shift in pace-of-life shift linked to urbanization is in general correspondence with expectations under a scenario of adaptive evolution

in response to the urban heat island effect, we do not preclude that other stressors linked to urbanization may equally have impacted this shift. A survey of local environmental conditions in a set of 83 ponds (Jessie Engelen, Caroline Souffreau and others, unpublished data; see also Gianuca *et al.*, 2018) did not reveal strong and systematic differences in nutrient concentrations (eutrophication), salt pollution, pesticide load and metal contamination along the urbanization gradient. Yet, our observation of a change in pace-of-life might reflect adaptation to a higher incidence of mechanical and hydrological disturbances (e.g. pond management: clearance of the litter layer, addition and removal of water) or extreme temperatures that are likely more prominent in urban compared to rural ponds (Wouters *et al.*, 2017; Brans *et al.*, 2018a; KIB personal observations).

Our study involved multiple genotypes isolated from a total of 13 populations along independent urbanization gradients in Flanders (Northern Belgium). The observation that the differentiation among rural and urban populations was highly repeatable, as illustrated by the population averages plotted in Fig. 3, indicates that the observed evolutionary changes are a general response to urbanization in this species in the study region (see also Johnson *et al.*, 2015; Thompson *et al.*, 2016; Tüzün *et al.*, 2017b,c). Our results on local adaptation at short spatial scales (Richardson *et al.*, 2014) are in line with previous studies providing evidence for adaptive genetic trait change to other anthropogenic disturbances in water fleas over short time scales (months to a few years) and spatial distances (Hairston *et al.*, 2005; Geerts *et al.*, 2015; Jansen *et al.*, 2015; Zhang *et al.*, 2016), and with our earlier results on the evolution of a higher thermal tolerance in city populations (Brans *et al.*, 2017b). We obtained evidence for clonal variation in genotypic trait values within populations for three out of eight measured traits in both urban and rural populations, indicating that both sets of populations harbor the capacity to respond in their life histories to further temperature increases either due to enhanced urbanization or to climate warming.

The common garden approach we used for testing genotypes at two temperatures, approximately corresponding to the temperature difference between urban and rural habitats (Brans *et al.*, 2018a, see also Fig. S1), allowed assessing relative contributions of evolution phenotypic plasticity, and evolution of plasticity to total trait change (Merilä & Hendry, 2014; Govaert *et al.*, 2016; Stoks *et al.*, 2016; Diamond & Martin, 2017) under an urbanization scenario. This scenario assumes that the urban heat island effect results in a temperature increase of approximately 4 °C, and that average summer temperatures in rural systems are around 20 °C (Brans *et al.*, 2017a; Brans *et al.*, 2018a; De Ridder *et al.*, 2015). Evolution accounted for a substantial portion (30%) of total observed multivariate phenotypic trait change (i.e. from rural populations at 20 °C to urban populations at 24 °C). Evolutionary divergence of city compared to rural populations was more important than phenotypic plasticity to a temperature difference of 4 °C (corresponding to the average temperature difference between city and rural ponds) of the ancestral population for size at maturity and number of juveniles in the first and second clutch, while plasticity in response to the 4 °C difference in rearing temperature

accounted for the majority of trait change observed in traits related to developmental rate, performance, and somatic growth. Plasticity is an important first crucial buffer against the impact of (anthropogenic) environmental change and habitat disturbance (Gienapp *et al.*, 2008; Donnelly *et al.*, 2012; Merilä & Hendry, 2014). It is becoming increasingly clear, however, that acclimatization alone might not guarantee long term population persistence under strong anthropogenic pressure (Visser, 2008; Franks *et al.*, 2014; Duputié *et al.*, 2015; Gunderson & Stillman, 2015). While phenotypic plasticity can feed evolutionary processes via genetic assimilation (Waddington, 1953; Lande, 2009; Ehrenreich & Pfennig, 2016), our study provides no direct evidence for a process that would turn phenotypic plasticity into genetic trait differentiation, given that overall, urban populations show the same univariate and multivariate plasticity responses to increased temperatures as rural populations, while they have strongly diverged in mean trait values.

Phenotypic plasticity responses to temperature increase were remarkably similar in urban and rural genotypes, except for size at maturity, for which we observed reduced plasticity in the city populations (see also Brans *et al.*, 2017a). Although this absence of evolution of plasticity contrasts to results on other systems and what is expected under anthropogenic disturbance scenarios (Reed *et al.*, 2011; Chevin & Hoffmann, 2017; Diamond & Martin, 2017), it might indicate there are constraints involved. For instance, we observe the strongest parallel responses for traits that are linked to rates (age at maturity and release of first and second clutch, somatic growth, performance). This may reflect the intrinsic relationship between metabolic rates and temperature (Brown *et al.*, 2004). The only trait for which we observe an urbanization x temperature interaction is size at maturity (urban animals here show reduced size-plasticity upon experimental warming compared to rural animals), which is also the trait for which we observe the strongest contribution of evolution. We hypothesize that this is due to the added selection for small body size imposed by the need to increase thermal tolerance (Geerts *et al.*, 2015; Brans *et al.*, 2017b), and that the reduced plasticity in urban animals is due to a trade-off associated with costs of very small body size at maturation.

As an increasing number of studies report genetic adaptation to urbanization in organisms ranging from plants (Cheptou *et al.*, 2008; Johnson *et al.*, 2015; Thompson *et al.*, 2016) over insects (Diamond *et al.*, 2017b; Tüzün *et al.*, 2017b) to birds (Atwell *et al.*, 2012; Mueller *et al.*, 2013), understanding and predicting how populations and species respond to regional and local increases in urbanization will require insights on evolutionary trait change. For all eight studied traits, trait changes that result from adaptation to urbanized settings are in the same direction as the plasticity-mediated responses to temperature increase (Fig. 4a). While the evolution of plasticity is in the opposite direction compared to evolution of mean trait value for four out of the eight traits (Fig. 4a), this component was small for all traits, and the evolution of mean trait value is for all traits in the same direction as the plasticity responses. This is in line with the idea that the urban heat island effect might be one of the important drivers of the observed evolutionary pace-of-life shift in urban *Daphnia*. We thus have no

evidence for thermal countergradient adaptation for life-history traits in this system (as was recently observed by Tüzün *et al.*, 2017b, for damselflies in the same region). As a result, the net change in trait value due to combined plasticity and evolution is large (Table S2, Supplementary Information B). For example, size at maturity changes from 2.87 mm to 2.63 mm (an 8.2% reduction), whereas age at maturity changes from 8.0 days to 5.2 days (a 34.8% reduction), and performance from 0.26 day^{-1} to 0.41 day^{-1} (a 56.2% increase). Given the additive nature of the net contribution of evolution (i.e. evolution of mean trait and of trait plasticity combined), it is also straightforward to calculate the additional 'gain' in a trait value that is achieved in urban populations in the warmer environment (24 °C) as a result of evolution. This gain is considerable, being approximately 10% for age at maturity and performance (as compared to the trait value in the absence of evolution). This may have important consequences of the functioning of urban ecosystems and species interactions. In this context, it is important to note that evolutionary trait changes might differ widely among organism groups in amplitude and direction. For instance, while Tüzün *et al.* (2017b) observed a countergradient response to the urban heat island effect in damselfly larvae, we here report a genetic response that is enhanced by the environmental effect of urban warming. As a result, evolutionary responses may either dampen or increase the degree of trophic mismatches along urbanization gradients, possibly impacting food web structure and dynamics (Clarke, 2003; Durant *et al.*, 2007; Visser, 2008; De Block *et al.*, 2013; Bonaviri *et al.*, 2017; Start *et al.*, 2017. The consequences of such evolution-driven mismatches have, however, not yet been quantified in the context of urbanization.

Strikingly, the genetic trait changes associated with adaptation to urban heat island and hot-tub effects that we report here (life-history traits) and in earlier work (Brans *et al.*, 2017b; thermal tolerance) broadly match observed genetic changes in relation to climate warming in *Daphnia* (Geerts *et al.*, 2015) as well as observed latitudinal variation in life-history traits (Sarma *et al.*, 2005) and thermal tolerance (Yampolsky *et al.*, 2014; Geerts *et al.*, 2015) in invertebrate ectotherms. Urbanization-induced genetic life-history changes show striking parallels with climate change-driven trait change during the past 60 years, with animals becoming smaller, maturing earlier, reproducing faster, and showing increased voltinism (Parmesan & Yohe, 2003; Daufresne *et al.*, 2009; Altermatt, 2010; Bestion *et al.*, 2015). This suggests that urban populations might be partially pre-adapted to climate change, which can profoundly impact spatio-temporal dynamics of species' responses to future climate change (Van Doorslaer *et al.*, 2009; Urban *et al.* 2012, 2016).

We conclude that urbanization strongly shapes pace-of-life evolution in *Daphnia magna*. Urban *Daphnia* mature earlier, have a smaller size at maturity, reproduce faster, produce larger clutches, and have a higher potential population growth rate when compared with rural populations. Univariate and multivariate thermal reaction norms show strong responsiveness to warming, and are similar in urban and rural populations. Plasticity responses upon warming and evolutionary trait shifts with increasing urbanization are in the same direction, which results in additive effects and pronounced trait changes as

one moves from rural population at rural temperatures (approximately 20 °C in the study region) to urban populations at temperatures simulating the urban heat island effect (here: 24 °C). The implications of the observed evolutionary responses can be manifold. For example, if in the absence of evolution these populations would not have been able to thrive in the urban environment (cf. evolutionary rescue; Gomulkiewicz & Holt, 1995), this would likely have a strong impact on the functioning of urban ponds, as large-bodied *Daphnia* are among the most efficient grazers on phytoplankton (Brooks & Dodson, 1965; Mourelatos & Lacroix, 1990; Gianuca *et al.*, 2016). The evolutionary shift to a faster pace-of-life and smaller body sizes may, however, also by itself lead to a reduced capacity of the *Daphnia* populations to top-down control algae (Goitom *et al.*, 2017). Finally, in the context of climate change, our results suggest that urban populations, in addition to already being adapted to higher temperatures, still harbor genetic variation that offers evolutionary potential for further adaptation to increased temperatures. In a more regional and subcontinental setting, it is possible that genetic adaptation to higher temperatures in urban areas might give urban populations an advantage over rural ones in the context of climate change, potentially resulting in urban populations re-invading rural settings. While the likelihood of such a scenario depends amongst others on the degree to which differences in urban and rural conditions, apart from temperature, would be more influential than pre-adaptation to higher temperatures, it is worth exploring its likelihood and potential consequences.

Acknowledgements

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Supplementary Information "City life on fast lanes: urbanization induces an evolutionary shift towards a faster life style in the water flea *Daphnia* "

SUPPLEMENTARY INFORMATION A: MATERIAL AND METHODS

Study organism and urbanization gradient

Urbanization was quantified as the percentage built-up area (BA, based on LRDgis maps available at Flanders Geographical Information Agency) at a radius of 3200 m around the pond (See Fig. 1 in the main text, and Table S1, Supplementary Information). During the summer of 2015, one additional population was found in the highly urbanized capital city of Brussels and was included in the experiment (population *Brxl*, Fig. 1 in the main text; see also Brans *et al.*, 2017b). Given that BA includes buildings, houses, and industrial infrastructure, but excludes road and parking lots, ponds situated in an area with a BA of 10% or more are regarded to be in a highly urbanized setting. We categorized ponds into 'urban' (BA > 10%; n = 8) and 'rural' (BA < 5%, n = 5) (Table S1, Supplementary Information; see also Gianuca *et al.*, 2016, who used the same categories in their analyses of community composition). Rural populations were characterized by an additional criterion of minimum 20% of Biologically Valuable Area in the regional surroundings of the pond (3200 m radius).

From each population, six females (four females for *Brxl*) were isolated to start up asexual clonal lines in the laboratory. *D. magna* is a cyclic parthenogen. This implies that females reproduce asexually under favorable conditions, enabling us to establish all-female clonal lineages that can be maintained in the laboratory for multiple generations. The monoclonal laboratory cultures were genetically screened using 27 microsatellite markers, following Jansen *et al.* (2011b) and Orsini *et al.* (2012) (detailed protocols can be found in Brans *et al.*, 2017b, Supplementary Information). Lines that were differentiated by at least one of the microsatellite markers from all other lines were regarded independent clonal lineages (i.e. 'clone' or MLG, multi-locus genotype; see Table S1 of this Supplementary Information), which was included in the statistical analyses as an additional level of within-population replication.

Purging maternal effects and culturing conditions

For each line (except for *Brxl*), six replicate cultures were established (i.e. 36 cultures per population), later to be randomly assigned to the two temperature treatments, with three replicates per temperature treatment (two replicate cultures per lineage for the *Brxl* population). All lines (n = 440) were cultured independently for at least two generations under standardized laboratory conditions: one individual in 200 ml dechlorinated tap water, 80% medium refreshment every two days, 20 °C, 14:10 L:D photoperiod, daily fed $1x10^5$ cells ml^{-1} of the green alga *Acutodesmus obliquus*. This was done to purge and randomize maternal and grandmaternal effects, so that differences among genotypes and

populations can unambiguously be assigned to genetic differences and evolutionary differentiation, respectively (Roff, 1992).

Statistical Analysis

MANOVA and linear mixed-effect models

To test for differentiation in life-history traits between urban and rural *Daphnia*, and between animals reared at 20 °C versus 24 °C, we first performed a MANOVA on all response variables scaled to a mean of 0 and standard deviation of 1 (fixed factors: urbanization, temperature treatment, and their interaction term). A random error term for clone was included into the model. Pillai's trace test statistic was extracted using the 'car' package (R Core Development Team, 2016). We tested for multivariate normality using the package 'mvtnormtest' (R Core Development Team, 2016). Number of juveniles in the second clutch was log-transformed to meet assumptions of normality.

Univariate linear mixed-effect model fittings (fixed effects: urbanization, temperature treatment, and their interaction term, random effects: population, nested in urbanization, and clone, nested in population; packages 'lme4' and 'car' were used to construct lmer models and generate Anova output tables) were performed as in Brans *et al.*, (2017b): F-tests, were fitted with the restricted maximum likelihood ratio; degrees of freedom were corrected using the Kenward-Roger approximation (Alnosaier, 2007). Assumptions of normality were checked by plotting model residuals versus fitted values, model residual histograms, normal Q-Q plots, and performing Shapiro-Wilk's tests on model residuals. Deviations regarding homogeneity of variances were tested via Levene's tests for homogeneity of variances with both treatment and urbanization level as grouping factors on each response variable. All assumptions were met, except for the variable 'number of juveniles in the second clutch', which was log-transformed in order to meet the assumption of normality.

Principal Component Analysis (PCA) and Phenotypic Trajectory Analysis (PTA)

We calculated 8-dimensional multivariate trait centroids for urban and rural populations at 20 and 24 °C. Centroids were calculated separately for each population x treatment combination, after which means per level of urbanization and temperature were assessed. We then tested for differences in the multivariate phenotypic plasticity trajectories (magnitude and angle), here given by a trait centroid in 8-dimensional traits space at 20 °C and 24°C, using the method described in Collyer & Adams (2007, see also Fig. 1a,b of the main text), i.e. via a residual randomization in which urbanization, treatment, and the interaction term were included in the statistical model including all observations. Following Stoks *et al.* (2016), we extended the model with population and clone as random factors (clone nested in population), which then allowed for testing differentiation in multivariate reaction norms and convergence or divergence via residual randomization, while still accounting for random and nested data structure. Secondly, in addition to comparing the magnitude and angle of reaction norms between

temperature treatments, we also performed the same analysis but comparing urban and rural groups within temperature treatments to assess convergence or divergence and genotypic multivariate differentiation (further referred to as 'evolutionary trajectories'). An evolutionary life-history shift between rural and urban centroids would be indicated by a vector that is significantly different form 0 in multivariate trait space (see also Fig. 1b of the main text).

To visualize the multivariate phenotypic plasticity and evolutionary trajectories, to reduce trait dimensionality, and to detect the covariation structure in the data that explain most of the observed trait variation, we conducted a principal component analysis (PCA), after which the scores on the varimax normalized axes were plotted (PC1-PC2, PC1-PC3; for all data points and for population and group centroids). Note that while PTA is conducted in the 8-dimensional trait space, the magnitude and angles of the multivariate trajectories are only visualized along bivariate projections plots of the principal components.

The relative contribution of univariate and multivariate evolution, phenotypic plasticity, and the evolution of plasticity, to trait change

Given that we measured an aquatic urban heat island effect of approximately +4 °C during summer (2015: +3.93 °C $T_{average}$,+4.03 °C T_{max}, Brans *et al.*, 2017a) between rural and urban ponds, and the average summer temperature in rural ponds is approximately 20 °C, the three components of trait change are computed as follows (according to Govaert *et al.*, 2016 and Stoks *et al.*, 2016; see also Fig. 1c,d of the main text): (i) 'ancestral phenotypic plasticity' (further referred to as '(thermal) plasticity') denotes the plasticity in trait value in the rural populations in response to a temperature increase of 4 °C, and is hence given by the difference in character states of rural populations when reared at 24 °C compared to 20 °C. It quantifies trait change when the rural population would be exposed to an increase in temperature corresponding to a change to an urban environment; (ii) genotypic differentiation (further referred to as 'evolution'; called 'consecutive evolution' in Stoks *et al.*, 2016) from rural to urban populations is the change in character state from rural to urban populations under the temperature regime experienced by rural populations (i.e. 20 °C), and (iii) 'evolution of plasticity', being the difference in reaction norm slopes of the two populations. It can be easily calculated as the 'rest' component of trait change after accounting for pure evolution and plasticity-driven trait change. This resulted in the following equations for all three components of trait change in a trait Y, with $\bar{y}$ the average trait value (obtained from the least-squares means output from each linear mixed-effect model including both fixed and random effects) in urban (index U) or rural (index R) populations at 20 °C or 24 °C (see also Fig. 1c of the main text):

$$Evolution = \bar{y}^{U}_{20\,°C} - \bar{y}^{R}_{20\,°C}$$

$$Plasticity = \bar{y}^{R}_{24\,°C} - \bar{y}^{R}_{20\,°C}$$

$$Evolution\ of\ plasticity = (\bar{y}^{U}_{24\,°C} - \bar{y}^{U}_{20\,°C}) - (\bar{y}^{R}_{24\,°C} - \bar{y}^{R}_{20\,°C})$$

Total phenotypic trait change was partitioned into each of these three components for all life-history traits separately, and for the integrated multivariate phenotypic trajectories. Applying this reaction norm approach in multivariate trait space is based on the same principles and similar formulae, but uses the population phenotypic centroids in the 8-dimensional trait space, calculated for each population when reared at 20 °C and 24 °C. Population centroid vectors were averaged for 'urban' and 'rural' populations, to obtain the 'urban' and 'rural' centroids at 20 °C and 24 °C in the 8-dimensional trait space. Multivariate evolution, phenotypic (thermal) plasticity, and evolution of plasticity are then 8-dimensional vectors, given by the following calculations (see also Fig. 1d of the main text):

$$Evolution = magn.^{U}_{20\,°C} - magn.^{R}_{20\,°C}$$

$$Plasticity = magn.^{R}_{24\,°C} - magn.^{R}_{20\,°C}$$

$$Evolution\ of\ plasticity = (magn.^{U}_{24\,°C} - magn.^{U}_{20\,°C}) - (magn.^{R}_{24\,°C} - magn.^{R}_{20\,°C})$$

The relative contributions of each component to the total observed univariate and multivariate change in life-history traits was obtained by dividing the absolute value of each component by the sum of the absolute values of the three components.

SUPLEMENTARY INFORMATION B: FIGURES AND TABLES

Figures

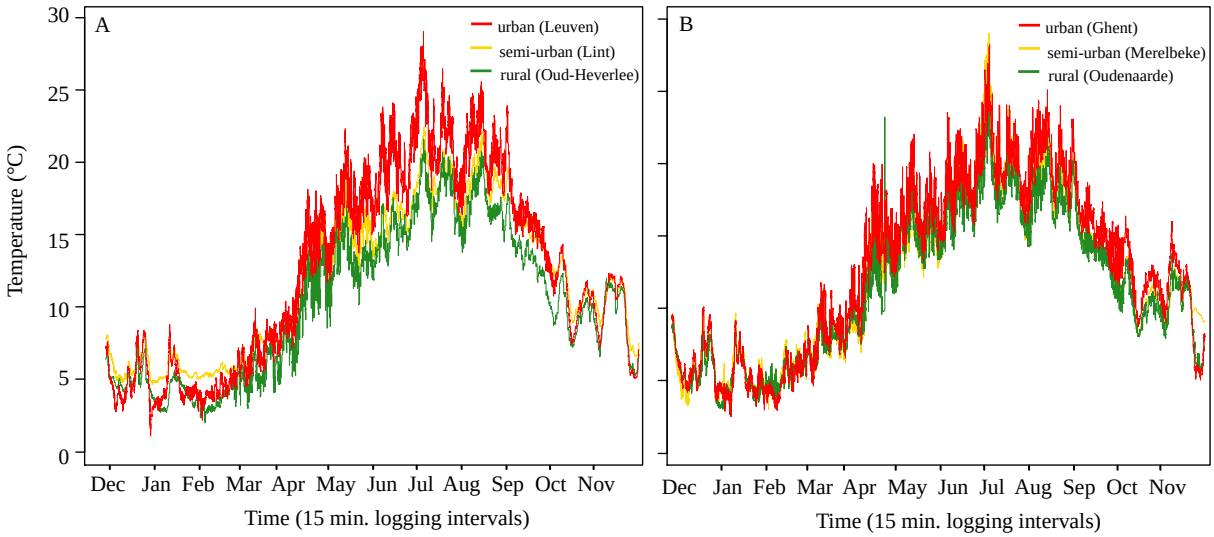

Figure S1. Temperature profiles (°C, measured at 15 min. intervals; period November 2014- November 2015) for an urban (red), semi-urban (yellow), and rural (green) pond in the regions of (A) Leuven and (B) Gent in Flanders, Belgium, in the eastern and western part of the region of Flanders, respectively (adapted from Brans *et al.* 2018a, *Landscape and Urban Planning*).

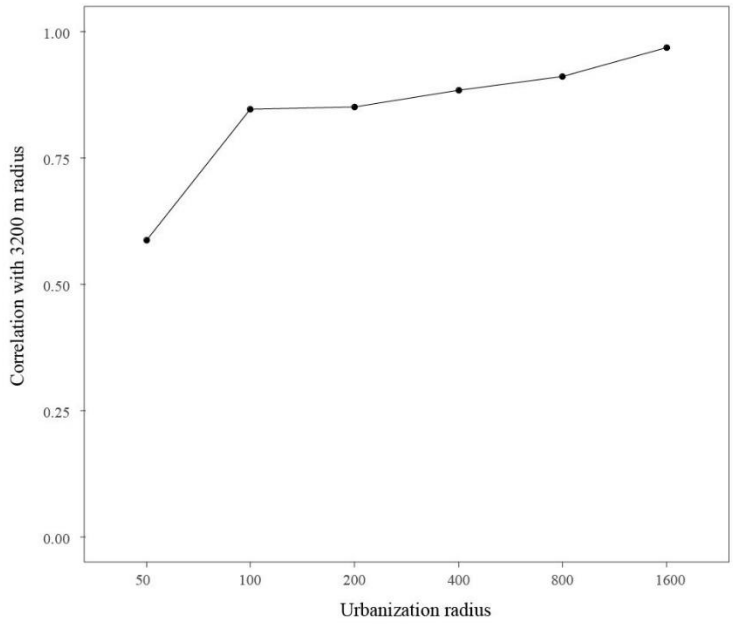

Figure S2. Correlations between urbanization level at different spatial scales (50 – 1600 m) with the urbanization level at 3200 m (the scale we used to select our study populations), plotted for each spatial scale. Urbanization was initially assessed at a 3200 m radius, given that this reflects regional urbanization levels. Later assessment of %BA at smaller spatial scales (50-100-200-400-800-1600 m radii) shows strong correlations with the level of urbanization at 3200 m from a radius of 100 m onwards, thus reflecting ponds were similarly 'urban' at relatively small (100 m) and larger spatial scales (3200 m).

Tables

Table S1. Locations of the *D. magna* study populations including the percentage built-up area (BA) in a radius of 3200 m around each pond, the according urbanization categorization, and the number of experimental lines and multi-locus genotypes (MLGs) used in the experiment.

Population		**Location**		**Urbanization**		**Exp. lines**
Pond	Code	Latitude (N)	Longitude (E)	%BA	Cat.	(MLGs)
Brussel	*Brxl*	50.855464	4.347334	40.440	urban	4(4)
Damme	*Damm*	51.26207	3.276039	1.126	rural	6(6)
Gent (Citadelpark)	*GenC*	51.03890	3.723744	24.242	urban	6(6)
Gent (Muinkpark)	*GenM*	51.04251	3.731611	25.736	urban	6(5)
Geraardsbergen	*Gera*	50.78420	3.915978	4.382	rural	6(3)
Kortrijk (Blauwe Poort)	*BppK*	50.81624	3.271563	15.356	urban	6(6)
Lapscheure	*Laps*	51.28253	3.355467	0.637	rural	6(5)
Leuven (Oude-Meren)	*OudM*	50.86328	4.723935	13.188	urban	6(5)
Mechelen (Kruidtuin)	*Mech*	51.02402	4.484039	14.125	urban	6(2)
Meerdaal (Zoete Waters)	*ZwMe*	50.82274	4.653691	2.017	rural	6(6)
Midden-Limburg (BKN1)	*MidL*	50.98233	5.317858	3.721	rural	6(5)
Sint-Niklaas (Bufferbekken)	*BuSN*	51.17808	4.161051	12.479	urban	6(4)
Sint-Niklaas (Fabiola Park)	*FaSN*	51.15623	4.159557	14.995	urban	6(5)

Table S2. Evolutionary gain calculated for each life-history trait. Percentages are given as an absolute value, accompanied by the direction of observed trait change, as well as the net phenotypic trait change from rural populations reared at 20 °C to urban populations reared at 24 °C (in unit specific measurements and as percentage of trait change.

Life-history trait	**% Evolutionary gain (absolute value)**	**Direction of trait change**	**Net phenotypic trait change (unit)**	**Net phenotypic trait change (%)**
Performance (*r*)	10.301	+	+ 0.15 d^{-1}	+ 56.186
Age at maturity	9.559	-	- 2.79 d	- 34.786
Release 1st clutch	7.371	-	- 3.23 d	- 32.214
Release 2nd clutch	6.448	-	- 3.85 d	- 29.153
Somatic growth	5.731	+	+ 0.06 d^{-1}	41.659
Size at maturity	5.096	-	- 0.23 mm	- 8.202
Number of juveniles in 1st clutch	4.985	+	+ 2.21	+ 26.276
Number of juveniles in 2nd clutch	4.346	+	+ 1.25	+ 10.646

Chapter 4

Urbanization drives genetic differentiation in physiology and structures the evolution of pace-of-life syndromes in the water flea *Daphnia magna*

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Abstract

Natural and human-induced stressors elicit changes in energy metabolism and stress physiology in populations of a wide array of species. Cities are stressful environments that may lead to differential selection on stress-coping mechanisms. Given that city ponds are exposed to the urban heat island effect and receive polluted run-off, organisms inhabiting these ecosystems might show genetic differentiation for physiological traits enabling them to better cope with higher overall stress levels. A common garden study with 62 *Daphnia magna* genotypes from replicated urban and rural populations revealed that urban *Daphnia* have significantly higher concentrations of total body fat, proteins, and sugars. Baseline activity levels of the antioxidant defense enzymes superoxide dismutase (SOD) and glutathione-S-transferase (GST) were higher in rural compared to city populations, yet urban animals were equally well protected against lipid peroxidation. Our results add to the recent evidence of urbanization-driven changes in stress physiology and energy metabolism in terrestrial organisms. Combining our results with data on urban life-history evolution in *Daphnia* revealed that urban genotypes show a structured pace-of-life syndrome involving both life-history and physiological traits, whereas this is absent in rural populations.

INTRODUCTION

Cities play host to one of the most remarkable shifts in species occurrence and loss, community structure, biotic homogenization, and rapid adaptive evolution (McKinney, 2008; Hahs *et al.*, 2009; Donihue & Lambert, 2015; Munshi-South *et al.*, 2016; Brans *et al.*, 2017a; Johnson & Munshi-South, 2017). Multiple stressors, such as pollution, drought, heat, and changed biotic interactions act in concert in cities (Parris, 2016), generating an environment that is thought to select for those species and phenotypes able to cope with higher overall stress levels (e.g. Partecke *et al.*, 2006; Bonier *et al.*, 2007; Isaksson, 2015; Meillère *et al.*, 2015; Hutton & McGraw, 2016).

Maintaining homeostasis depends heavily on the balance between oxidative damage and antioxidant repair mechanisms (Korsloot *et al.*, 2004; Costantini, 2014). Oxidative damage to lipids, proteins, and DNA can be caused by pro-oxidative by-products of respiration (reactive oxygen and nitrogen species, ROS and RNS; Costantini, 2014). This leads to oxidative stress, a state in which cellular antioxidant and repair mechanisms fail to quench excess levels of ROS/RNS (Korsloot *et al.*, 2004, Costantini, 2014). Natural stressors, such as predation, overcrowding (competition), food stress (low food quantity or quality), oxygen depletion, and temperature extremes are known to cause enzymatic and oxidative trait changes linked to stress resistance (Di Giulio *et al.*, 1989; Janssens & Stoks, 2013; Costantini, 2014; Chainy *et al.*, 2016; Burraco *et al.*, 2017). In cities, these environmental stress factors are disproportionally altered due to human activity (Parris, 2016). Additionally, non-natural perturbations linked to pollution (e.g. metals, pesticides, noise, and light pollution) have been shown to change redox states in terrestrial and aquatic animals due their high oxidative reactivity with biomolecules (Hoffman, 2002; Demirel *et al.*, 2009; Isaksson, 2010; Sniegula *et al.*, 2017). Therefore, it might be expected that city populations feature adaptive changes in antioxidant defense mechanisms (Møller *et al.*, 2010; Costantini *et al.*, 2014; Isaksson, 2015).

Studies on stress physiology of urban species and populations have only started to emerge, but evidence is rapidly growing that urbanization has a significant impact on the regulation of oxidative stress machinery (e.g. antioxidant enzymes such as superoxide dismutase -SOD, catalase -CAT, and glutathione-S-transferase -GST), immune function, stress-related behavioral changes, and energy metabolism (Partecke *et al.*, 2006; Falfushinska *et al.*, 2008; Møller *et al.*, 2010; Giraudeau & McGraw, 2014; Herrera-Dueñas *et al.*, 2014; Isaksson, 2015; Hutton & McGraw, 2016; Herrera-Dueñas *et al.*, 2017). For example, field-caught urban house sparrows have higher activity of antioxidant enzymes (SOD and GST), yet lower total antioxidant capacity (TAC) and higher levels of oxidative damage in their blood compared to rural birds (Herrera-Dueñas *et al.*, 2017). Additionally, energy reserve compounds such as fat, sugars, and proteins can be an important component of stress responses, as they provide cellular energy that can be allocated to growth and reproduction, active dispersal, or an increase in physiological stress responses, and thereby provide the fuel to cope with stressful conditions and

enable population survival (Sokolova, 2013; Meillère *et al.*, 2015). For instance, field-caught juvenile house sparrows contain higher levels of body fat in urban than in rural areas (Meillère *et al.*, 2015).

Small aquatic ecosystems such as ponds located in the city are warmer (Brans *et al.*, 2017a, 2018a) and often receive organic and chemical pollutants such as road salts, nutrients, metals, and pesticides via surface run-off or atmospheric depositions (Hassall, 2014; Jones *et al.*, 2017). In addition, they are often characterized by higher disturbance levels (e.g. water level control, leaf litter cleaning; Hassall, 2014; personal observations). As populations of plants and animals inhabiting urban ponds might be exposed to greater environmental stress, we hypothesize that urban environments select for different coping mechanisms at the physiological level, causing genetic differentiation between rural and urban populations in traits such as energy storage molecules (e.g. fat, proteins, and sugars), and oxidative stress enzymes (CAT, SOD, GST). Although some studies observed clear phenotypic upregulation of stress physiological parameters (e.g. GST: Crago *et al.*, 2011; Hsp70: Ravaschiere *et al.*, 2017), evidence for an evolutionary shift in organismal stress physiology in cities is so far limited (but see Partecke *et al.*, 2006; Costantini, 2014).

To quantify the degree of urbanization-driven genetic differentiation in stress physiology, we set out a common garden experiment with urban and rural genotypes of *Daphnia magna*, originating from replicated urban and rural populations (13 populations located across well-characterized urbanization gradients in Flanders, Northern Belgium). *Daphnia* from city populations mature faster and at a smaller size, produce more offspring and release their juveniles sooner, and are characterized by a higher intrinsic population growth rate (Brans & De Meester, 2018; Fig. S3, Supplementary Information A). In our common garden experiment, we measured seven physiological response variables that are either linked to energy metabolism (total fat, sugar, and protein content) or oxidative stress responses, quantifying both antioxidative defense (activity of SOD, CAT, and GST) and damage (concentration of malondialdehyde, a by-product of lipid peroxidation). We reared animals at two different temperatures (20 and 24 °C) to mimic the urban heat island reported to occur in these ecosystems (urban ponds are characterized by a higher mean, maximum, and minimum daily temperature compared to rural ponds, and this 'urban hot-tub effect' is most pronounced in summer; $\Delta T_{mean\text{-}summer}$ = 3.04 °C; $\Delta T_{max\text{-}summer}$ = 3.69 °C; Brans *et al.*, 2017a, 2018a; Fig. S1, Supplementary Information A), enabling us to quantify phenotypic plasticity across two temperatures and assess habitat-specific (urban versus rural) phenotypic plasticity (to warming) in the measured stress physiological responses.

We first tested the hypothesis that there is genetic differentiation between urban and rural *Daphnia* populations for these physiological responses. More specifically, we expected that urban *Daphnia* might be characterized by higher amounts of storage molecules that can be invested in faster growth and development, or in detoxification processes. Stress physiological responses might include

higher activity of antioxidant defense enzymes (SOD, CAT, GST), while suffering a similar or even reduced level of oxidative damage. We additionally tested for covariation patterns to explore to what extent the studied life-history and physiological traits are shaped by an overall pace-of-life syndrome (Réale *et al.*, 2010). The Pace-of-life Syndrome (POLS) hypothesis (Réale *et al.*, 2010) predicts a concerted change in both life-history and physiological traits, as the physiological traits determine how much energy can be invested in life-history features (Ricklefs & Wikelski, 2002). The presence and structure of covariation patterns has been suggested to be environment-dependent and potentially under selection (e.g. Killen *et al.*, 2013; Charmantier *et al.*, 2017). Using multi-group structural equation modelling (SEM) on urban and rural *Daphnia*, we tested whether pace-of-life-syndrome covariation patterns are dependent on urbanization background (evolution) or rearing temperature (plasticity) (Niemelä *et al.*, 2013; Debecker *et al.*, 2016; Tüzün *et al.*, 2017c). More specifically, we hypothesize that especially in urban populations, a close link between life-history and physiology might have evolved, where physiological processes (e.g. energy budget) are aligned to support their faster life style.

MATERIAL AND METHODS

Urbanization gradient, model organism, and experimental design

We sampled 13 *Daphnia magna* populations along multiple well-characterized urbanization gradients in Flanders (Northern Belgium; see Fig. S2, Supplementary Information A), with the percentage built-up area (BA; available via the Large-scale Reference Database at Flanders Geographical Information Agency) in the regional surroundings of the pond (3200 m radius) as a proxy for the degree of urbanization. Given that this measure does not include roads and parking lots, an area with a BA of 10% and more is considered highly urbanized. We classified all ponds according to their %BA into rural (< 5%, n =5) and urban (> 10%; n = 7). Rural ponds were additionally characterized by a minimum of 20% of Biologically Valuable Area (3200 m radius) to ensure that they would be situated in natural areas and not in areas characterized by intensive agriculture. We performed all experiments with clones of these 13 populations, but due to accidental thawing we lost the set of samples of highly urbanized population *Brxl*, which prevented assessing physiological traits for this population.

Daphnia magna has a cyclic parthenogenetic life cycle; under favorable conditions all-female clonal lineages can be established and cultured over several generations. Six females from each population were isolated and reared under standardized laboratory conditions (dechlorinated tap water, 80% water replacement every two days, 20 °C, 14:10 L:D photoperiod, daily fed 10^5 cells.ml^{-1} of the green algae *Acutodesmus obliquus*; for more detailed information on culturing conditions we refer to Brans *et al.*, 2017a,b; and Supplementary Information B). After one generation of culturing under standardized conditions, 2nd brood juveniles from each line were split up and divided over six replicated cultures (i.e. 36 cultures per population, total number of experimental units: 432), later to be assigned

to the appropriate temperature treatment. We screened clonal identity of all lines using microsatellite markers (27 markers, detailed information in Brans *et al.*, 2017a), which was added as an additional level of within-population variation in the statistical analysis (i.e. 'clone', Table S1, Supplementary Information A). All population x line x temperature replicates were cultured for an additional two generations under the same standardized laboratory conditions to purge (grand)maternal effects, after which we transferred a cohort of 12 juveniles (< 24h old) of each line to the appropriate temperature treatment (20 °C/ 24 °C).

We carried out a cohort life table experiment with all lineages at two temperatures, 20 and 24 °C (constant temperatures), reflecting the average summer temperature in rural and urban populations, respectively (Brans *et al.*, 2018a; see Supplementary Information A, Fig. S1). Cohorts were followed up to assess thermal tolerance and measure life-history traits (reported in Brans *et al.*, 2017b, 2018b). After release of the 2nd clutch, six animals from each experimental cohort were pooled and flash frozen in liquid nitrogen until sample processing. Body length (from center of eye to base of tail spine) of three animals of each pooled sample was measured (Olympus X stereo microscope), and average length was translated into dry mass using a published length-weight regression (Dumont *et al.*, 1975) so as to standardize measured amounts of proteins, fat, carbohydrates and MDA by dry weight.

Quantification of response variables

The pooled sample of animals per replicate of a clonal line-by-treatment combination was homogenized with a pestle in PBS buffer (Phosphate-Buffered Saline, 50 mM, pH: 7.4, 50 µl/animal) and centrifuged for 8 min at 13000 rpm and 4 °C. Detailed sample analysis protocols are given in Supplementary Information (section B). In brief, total protein content was quantified following the Bradford protocol (Bradford, 1976), total fat content following Bligh & Dyer (1959), and total sugar content (glucose + glycogen) following Stoks *et al.* (2006).

The activity of superoxide dismutase (SOD), catalase (CAT), two key antioxidant enzymes in invertebrates (Korsloot *et al.*, 2004), and gluthation-S-transferase (GST), a secondary antioxidative enzyme that protects against oxidizing and toxic substances by detoxifying ROS-damaged cellular components, was measured as an estimate of antioxidant defense. SOD and CAT activity was quantified using the protocol of De Block & Stoks (2008), while GST activity was quantified following McLoughlin *et al.* (2000).

Oxidative damage was quantified as malondialdehyde (MDA) concentration (Grotto *et al.*, 2009), a byproduct of lipid peroxidation, following Debecker (2017) (total fat content was used as a covariate in the statistical analysis).

Statistical analysis

To test for differentiation in physiological traits between urban and rural *Daphnia* (categorical), and between animals reared at 20 °C and 24 °C (categorical), we first performed a MANOVA, followed by separate linear mixed-effect models using the R software (R Development Core Team, 2016; packages 'car', 'lme4', 'mvnormtest'), on all response variables scaled to a mean of 0 and standard deviation of 1 (fixed effects: urbanization, temperature, and their interaction). Population and clone within populations were implemented as random factors (clone nested in population, population nested in urbanization). Model specifications, random structure, computed test statistics, and the validation of model assumptions are detailed in Supplementary Information (section B). Significance of 'clone' reflects evolutionary potential within populations.

We tested for the impact of urbanization and/or temperature on covariation between life-history and physiological traits along the pace-of-life axis (cf. Pace-of-life Syndrome Hypothesis; Réale *et al.*, 2010) by combining the stress physiological traits with data on eight life-history traits measured on the same cohorts of individuals in a life table experiment (Brans & De Meester, 2018). *Daphnia* genotypes from urban populations show an evolutionary shift in life style towards the fast end of the pace-of-life continuum compared to *Daphnia* genotypes from rural populations (see Fig. S3, Supplementary Information A). Using structural equation modelling (Grace, 2006), we explored to what extent the shift towards a faster life style in urban populations covaried with trait shifts in stress physiology according to an overarching pace-of-life syndrome (Réale *et al.*, 2010), and whether urbanization (genetic differentiation) and/or temperature treatment (plasticity) shaped these POLS trait covariation patterns in a consistent manner (Dingemanse *et al.*, 2010; Tüzün *et al.*, 2017c).

Using multi-group SEM (Shipley, 2009), we first evaluated the consistency of trait covariation patterns at both levels of biological organization separately (i.e. life-history trait covariation patterns and physiology trait covariation patterns, independent of each other; paths a-i and j-p, respectively, in Fig. S4, Supplementary Information A). Second, we evaluated consistency of trait variation in an overarching pace-of-life syndrome model integrating life-history and physiology (paths q and r, Fig. S4). The rationale behind each SEM is that a 'latent' (i.e. non-measured) variable can be incorporated in the model (Fig. S4, ovals) that structures the covariation patterns observed in the measured variables (squared boxes). The suits of traits linked in a 'syndrome' given by the latent variable are indicated via significant pathways (Grace, 2006). Each pathway in the SEM represents a linear model. For significant paths, a change in one variable (standardized units for all variables) coincides with a change in all other traits of the syndrome (according to path coefficients, which represent standardized model estimates). Multi-group SEM allows to compare models with factor loadings that are constrained or freely varying across temperature treatment, urbanization level, or a combination of both (detailed information in Supplementary Information B). This results in different model hypotheses (Model I to IV, Table S2,

Supplementary Information A). For example, if the best model fit is characterized by factor loadings being constrained across temperature treatments, but not urbanization level (model II), this indicates that trait covariation patterns significantly differed between urban and rural populations but were the same for animals reared at 20 and 24 °C (and vice versa for model III). This would imply that urbanization background, and thus evolution (or temperature and thus plasticity, for model III), significantly shaped trait covariation patterns. Models were ranked according to AIC scores, model weights (W), and evidence ratios (E.R.) (detailed in Supplementary Information, section B). When models II, III, or IV were selected as the best model (i.e. a non-fully restricted model), differences between groups were tested by comparing factor loadings (significance, direction, and size) using the method of Zar (1999) (see also Dingemanse *et al.*, 2010; Debecker, 2017; Tüzün *et al.*, 2017c; and Supplementary Information section B for calculations). All models were fitted with the package 'lavaan' (Rosseel, 2012). All variables were scaled and standardized prior to analysis.

RESULTS

Genetic differentiation in energy reserves and stress physiology

MANOVA indicated an overall significant effect of urbanization on the combined set of physiological response variables ($p < 0.001$, Table 1). General linear-mixed effect models on each variable separately revealed that urban animals contained significantly more fat ($p = 0.003$, Fig. 1a), sugars ($p < 0.001$, Fig. 1b), and protein ($p = 0.002$, Fig. 1c) per µg dry mass (Table 1), suggesting a higher amount of energy reserves in urban compared to rural populations. Animals reared at a higher temperature had less protein per µg dry mass ($p = 0.02$, Table 1) but did not show differences for fat and sugar content (Table 1).

Urban animals did not differ from rural ones in CAT activity ($p = 0.525$, Fig. 2a), but had a lower SOD ($p = 0.013$, Table 1, Fig. 2b) and GST ($p = 0.023$, Table 1, Fig. 2c) base line activity. MDA concentrations (lipid peroxidation) did not differ between urban and rural animals ($p = 0.835$, Fig. 2d). Elevated rearing temperature induced a significant increase in the activity of CAT ($p < 0.001$, Fig. 2a) and SOD ($p < 0.001$, Fig. 2b), but not in GST, and there were no differences in lipid peroxidation. We did not observe significant urbanization x temperature interaction effects for any of the traits.

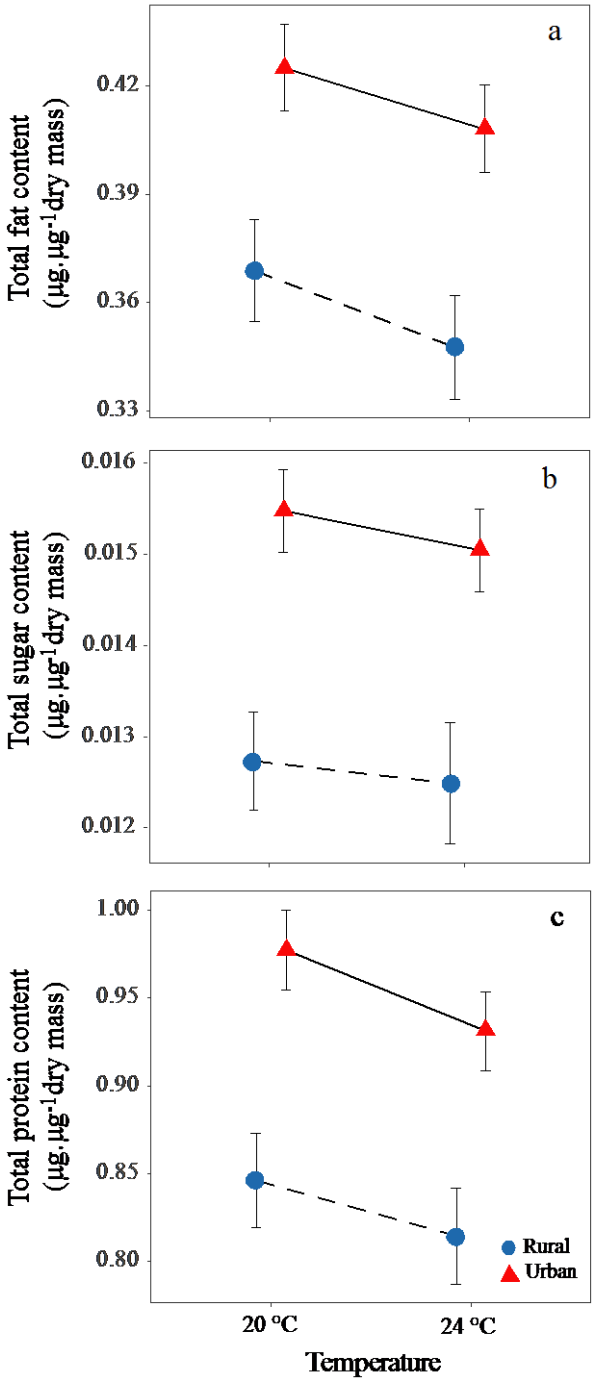

Figure 1. Energy storage compounds. Mean ± 1 SE fat (a), sugar (b), and protein (c) content for urban (triangles) and rural (circles) animals reared at 20 °C and 24 °C. Significant differences between urban and rural animals are given by colored symbols (urban: red; rural: blue).

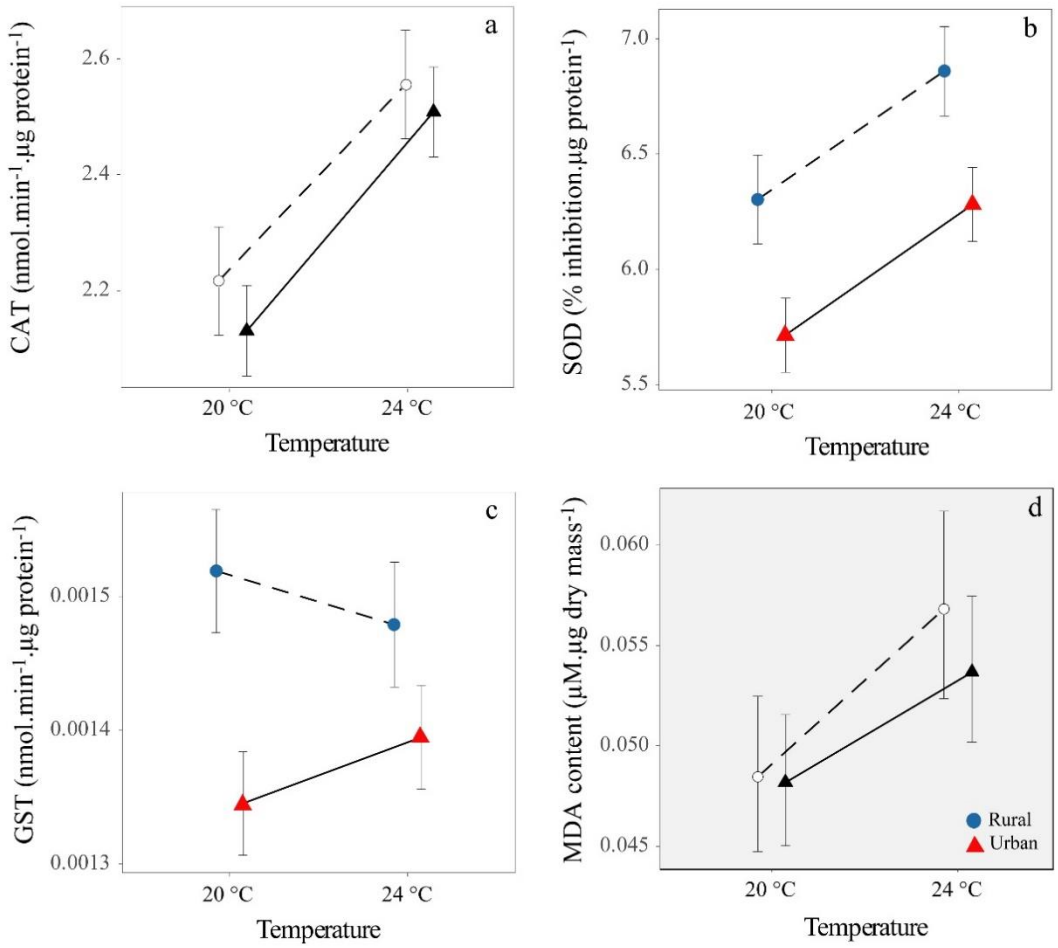

Figure 2. Antioxidant enzymes (a-c, white) and oxidative damage (d, grey). Mean ± 1 SE CAT activity (a), SOD activity (b), GST activity (c), and lipid peroxidation, measured as MDA (d) for urban (triangles) and rural (circles) animals reared at 20 °C and 24 °C. Significant differences between urban and rural animals are given by colored symbols (urban: red; rural: blue). Non-significant differences for the effect of urbanization are depicted in black/white.

Structural equation modelling: exploring the Pace-of-life Syndrome Hypothesis

Life-history trait covariation patterns

The path analysis on life-history traits revealed a syndrome structure with underlying life-history trait covariation differing according to model IV (Table S3, factor loadings all free; Fig. S5, Supplementary Information A), indicating differently structured life-history trait covariation patterns across all four urbanization x temperature treatment groups. This implies that both urbanization background (evolution) and temperature treatment (plasticity) significantly shape life-history trait covariation (more detailed results are given in Supplementary Information C).

Physiology trait covariation patterns

The best model explaining a structuring syndrome at the level of physiological traits was model I (Table S3, all factor loadings constrained to be equal: Fig. S6, Supplementary Information A), indicating a

consistent trait covariation across all four urbanization x temperature groups (more detailed results are given in Supplementary Information C).

Overarching Pace-Of-Life Syndrome

In the overarching POLS models, model IV was the best supported, indicating that covariation patterns differ among all groups (Table S3). Both urbanization background (genetic differentiation) and temperature treatment (plasticity) shape trait covariation patterns. The difference in trait covariation patterns between urban and rural animals reared at 20 °C reflects genetic differentiation for a POLS (Fig. 3a,b; significantly differing paths at the level of $p < 0.05$ are indicated with †; for the pattern at 24 °C, see Fig. S7, Supplementary Information A). The major difference between urban and rural animals at 20 °C was that the overarching syndrome structure linking the physiological and life-history syndromes was significant in the urban but not rural animals (Fig. 3a,b; paths q and r in Fig. S4, Supplementary Information A; both $p < 0.001$). This overarching POLS reflects that, within the set of urban animals, fast-paced (i.e. fast maturation, high fecundity, high population growth rate) animals have lower amounts of protein, fat, and sugars, but higher activity of oxidative stress enzymes and higher oxidative damage. This suggests that, within urban animals, shifts towards a faster life-style depletes energy resources and increases oxidative stress physiology. In the rural path model, no such link between physiology and life history, and thus no overarching POLS is present (Fig. 3b). Within the nested life-history syndrome, fecundity (number of juveniles in both clutch 1 and 2) significantly contributed to the syndrome structure in rural but not in urban animals (both $p < 0.001$, Fig. 3a,b). When looking at the nested physiological syndrome, MDA significantly covaried with other physiological traits in urban but not in rural animals ($p < 0.001$, Fig. 3a,b), whereas GST covaried with other physiological variables in rural but not in urban animals ($p = 0.049$, Fig. 3a,b). Of the significant paths shared between both covariation models (i.e. paths significantly contributing to the syndrome structure in both urban and rural animals), all path coefficients had the same direction in both groups, but significantly differed in size between urban and rural genotype sets for development time (age at maturity, age at release of first and second clutch), fecundity (both clutches), somatic growth, total protein content, MDA, and SOD (Fig. 3a,b; indicated with †, $p < 0.05$).

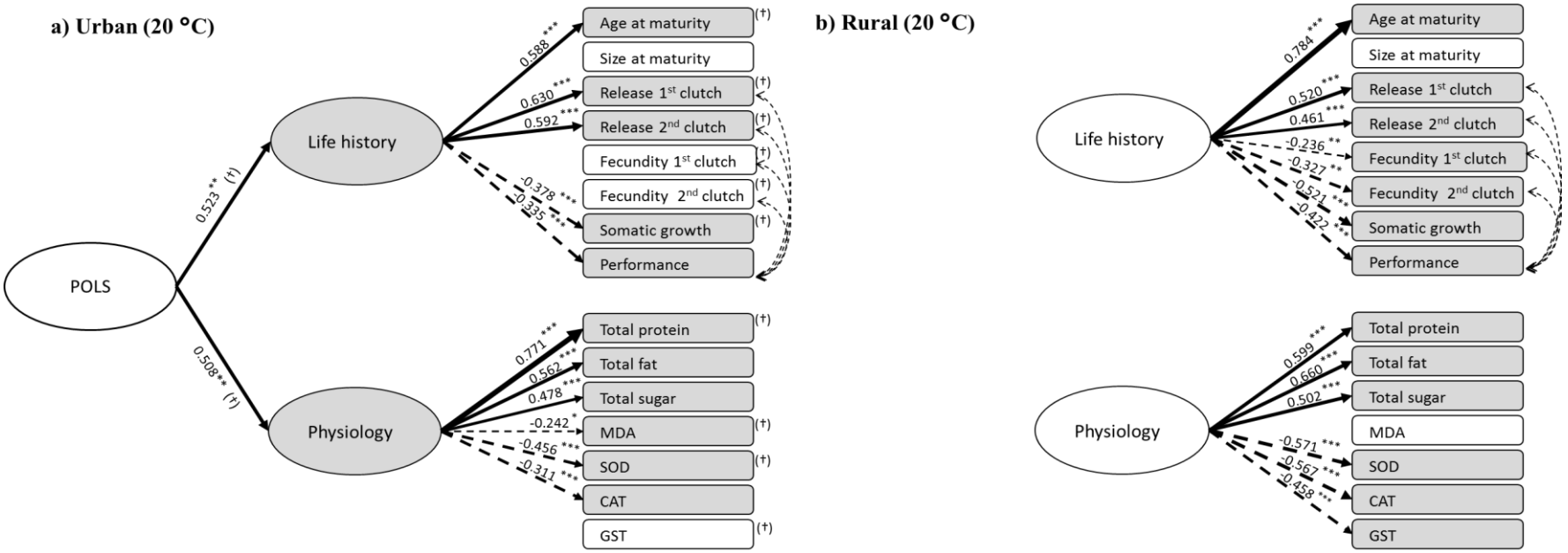

Figure 3. Structural equation models. Path models showing the covariation pattern of life-history and physiological traits (i.e. measured traits, boxes) within a life-history, physiology, and overarching pace-of-life syndrome (POLS) (all modeled as latent variable, ovals) in a) urban and b) rural genotype sets of *Daphnia magna* when reared at 20 °C. Differences in covariation structures between the two groups reflect genetic differentiation. Only paths significantly contributing to syndrome structure (i.e. significantly different from 0) are depicted (associated traits are in grey colored boxes), and accompanied with path coefficients. Significance levels of path coefficients are depicted as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Path coefficients indicate the change (in SD units) of traits predicted to occur based on a 1 SD change in the underlying syndrome structure. Arrow thickness (dashed arrows: negative correlations, full arrows: positive correlations) is proportional to the strength of the path loading. Path loadings significantly differing between urban and rural genotype sets (either because of their significant contribution to a syndrome in one group, but not in the other, or because of a significant difference in direction or size of path loadings that significantly contribute to syndrome structures in both groups) are indicated with † ($p < 0.05$ after Bonferroni correction). Dashed double-headed arrows indicate correlated errors.

Table 1. Multivariate and univariate statistical results. Results of the MANOVA and univariate linear mixed-effect models, testing for the effect of urbanization level, treatment, and their interaction on a set of seven physiological parameters linked to energy metabolism (total protein, fat, and sugar content) and oxidative stress (antioxidant enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase (CAT); oxidative damage: lipid peroxidation (MDA)), measured in a set of urban and rural *Daphnia magna* genotypes grown under common garden conditions. Total fat content was used as a covariate in the MDA model. Significant results ($p < 0.05$) are given in bold or indicated with "·" ($p < 0.1$). Numerator and denominator degrees of freedom are given by ndf and ddf respectively.

	Urbanization				**Temperature**				**Urbanization x Temperature**				**Clone (random)**	
Manova	**F**	**ndf**	**Pillai**	**p**	**F**	**ndf**	**Pillai**	**p**	**F**	**ndf**	**Pillai**	**p**		
All traits	7.820	7	0.543	**< 0.001**	1.230	7	0.158	0.306	1.022	7	0.134	0.429	*Included as random error term*	
Univariate anovas	**F**	**ndf**	**ddf**	**p**	**F**	**ndf**	**ddf**	**p**	**F**	**ndf**	**ddf**	**p**	χ^2	**p**
Total protein content	16.265	1	10.090	**0.002**	5.486	1	353.07	**0.020**	0.174	1	353.06	0.677	2.648	0.050·
Total fat content	14.671	1	9.68	**0.003**	2.985	1	352.40	0.085·	0.038	1	352.40	0.846	8.102	**0.002**
Total sugar content	23.0736	1	9.980	**< 0.001**	1.166	1	351.18	0.281	0.068	1	351.28	0.794	9.5027	**0.001**
MDA (lipid peroxidation)	0.045	1	10.68	0.835	0.579	1	355.25	0.447	0.238	1	354.37	0.626	0.106	0.372
SOD	8.808	1	9.85	**0.014**	12.779	1	358.84	**< 0.001**	0.001	1	258.84	0.973	2.598	0.054·
CAT	0.435	1	10.01	0.525	29.439	1	356.850	**< 0.001**	0.077	1	357.080	0.781	3.223	**0.037**
GST	7.282	1	9.79	**0.023**	0.141	1	357.89	0.707	1.419	1	358.01	0.243	5.447	**0.010**

DISCUSSION

The results of our common garden experiment reveal pronounced genetic differences between urban and rural populations of the water flea *Daphnia magna* for a set of physiological traits linked to stress resistance and energy reserves. Compared to rural populations, urban *Daphnia* have higher concentrations of protein, fat, and sugars, reflecting a higher level of energy storage compounds available per unit dry mass. The activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione-S-transferase (GST) were upregulated in rural animals, yet malondialdehyde (MDA) concentrations, a measure for lipid peroxidation and thus oxidative damage, did not differ between both genotype sets. These results suggest that urban *Daphnia* have evolved a more efficient stress resistance compared to rural animals. While raising the animals at 24 °C compared to 20 °C led to a reduction in total protein content and an increased activity of SOD and GST, we observed no urbanization x temperature interactions. Evolutionary differentiation thus involved constitutive trait changes but no evolution of plasticity. Integrating our data on physiological traits with existing data on life-history traits measured on the same set of genotypes reveal an overarching pace-of-life syndrome, structuring covariation patterns between life history and physiology in urban, but not in rural animals. Our results thus show that urbanization not only induces evolution of mean trait values, but also shapes trait covariation patterns. More specifically, in urban animals life history and physiology are integrated into a syndrome, covarying along the pace-of-life axis, while the two sets of traits were unlinked in rural animals. In the following paragraphs we discuss these results in more detail.

Genetic differentiation in energy reserves and stress physiology

Most urban ecological studies reporting on stress physiology focus on terrestrial vertebrates such as birds and mammals (Falfushinska *et al.*, 2008; Møller *et al.*, 2010; Giraudeau & McGraw, 2014; Herrera-Dueñas *et al.*, 2014; Hutton & McGraw, 2016; but see Falfushinska *et al.*, 2008 for amphibians). As most studies use field collected animals, they do not reveal whether or not the observed differences in physiological stress-coping mechanisms between urban and rural populations are driven by evolution (but see e.g. Mueller *et al.*, 2013; Harris & Munshi-South, 2017). Here we show that populations of the water flea *D. magna* inhabiting small ponds in urban areas have genetically diverged from those in rural areas. Urban *Daphnia* genotypes have higher concentrations of protein, fat, and sugars per unit of dry mass. Urban populations are therefore characterized by a higher available energy budget (De Coen & Janssen, 2003) than rural populations, which is important in determining their scope of growth under stress (Barata *et al.*, 2002; De Coen & Janssen, 2003). Urban populations are also characterized by a reduced activity of antioxidant enzymes (SOD and CAT), while oxidative damage to lipids did not differ, indicative of more effective defense responses or investment in other defense proteins. In general, these results are in line with earlier findings on the evolutionary and acclimatory responsiveness of *Daphnia* when exposed to human-associated toxicants such as heavy metals and

pesticides (Jemec *et al.*, 2012; Coldsnow *et al.*, 2017) and natural toxicants such as cyanotoxins (Sarnelle & Wilson, 2005).

The results of our study reinforce earlier observations on genetic differentiation in *D. magna* populations in response to urbanization, which involved both heat tolerance (Brans *et al.*, 2017b) and life-history traits (Brans & De Meester, 2018). Combined, they suggest that urban populations of *D. magna* are genetically different from rural ones in multiple traits linked to warming, pace-of-life, and overall stress. Traits range from heat tolerance (i.e. survival at higher temperatures) to energy metabolism, oxidative stress responses, and a diverse set of life-history features. The number of studies unequivocally showing evolution in response to urbanization is rapidly growing (e.g. Cheptou *et al.*, 2008; Thompson *et al.*, 2016; Brans *et al.*, 2017a,b; Diamond *et al.*, 2017b), but none involved as an extended set of traits as reported for *Daphnia.*

The Pace-of-life syndrome Hypothesis: linking life history and physiology

Our earlier work on life-history differentiation indicated that urban *D. magna* populations showed a clear-cut shift to a faster pace-of-life compared to rural populations. Due to energy allocation trade-offs, stress physiological responses are often closely intertwined with higher-level response traits such as behavioral or life-history traits, potentially leading to a pace-of-life syndrome (Réale *et al.*, 2010) structuring covariation patterns between life-history and physiological traits. Strikingly, structural equation modeling revealed such an association between physiological and life-history traits in urban but not in rural populations. In urban populations, animals with a faster life style, have a lower protein content, upregulated SOD and GST activity, and suffer higher lipid peroxidation (Fig. 3). These covariation patterns suggest that, within the set of urban genotypes, investment in a faster life style depletes energy reserves (see Fig. S8, Supplementary Information A) and increases antioxidant defense activity (SOD), yet still comes at a cost of increased lipid peroxidation (MDA). The fact that an underlying syndrome between life history and physiology is only present in the city populations is in line with the hypothesis that such strong associations between suits of life-history, physiological or behavioral traits are more likely to occur in low-quality habitats or stressful conditions (e.g. Bell & Sih, 2007; Killen *et al.*, 2013; Charmantier *et al.*, 2017; Tüzün *et al.*, 2017a).

While within urban genotypes the POLS reflects that a faster life style comes at a cost of reduced energy reserves and higher oxidative damage, we observe the opposite relation when comparing mean traits of urban and rural populations. Here, the overall mean faster life style of urban populations is associated with higher investment in energy storage (see also Conover & Schultz, 1997) and with reduced investment in antioxidant defense. Similar to the observations made in many other studies (reviewed in Meillère *et al.*, 2015), the evolution of oxidative stress responses in urban populations is not consistent across all three enzymes tested (Table 1; Fig. 2). Overall, however, there is a reduction in oxidative stress enzyme activity in urban compared to rural populations, while there is

no difference in oxidative damage, suggestive of a more efficient defense mechanism (Fig. 2). These results are at first sight counter-intuitive given that urbanization induces a faster pace-of-life, which is expected to result in a higher metabolic rate and associated increase in oxidative stress (Ricklefs & Wikelski, 2002; Angilletta, 2009). Our results might, however, reflect that urban populations are adapted to an overall more stressful environment through a reduced metabolic rate and investment in other defense (e.g. Heat Shock Proteins) proteins under baseline conditions, as has been repeatedly observed in a variety of organisms (Angilletta, 2009, Oksala *et al.*, 2014). This might be achieved through the evolution of more efficient defense mechanisms or through trade-offs with other traits, such an antipredator defense or immune function (Pauwels *et al.*, 2010; Rauw, 2012; Oksala *et al.*, 2014). Within the set of urban genotypes, however, variation in traits among genotypes does reflect a classic trade-off between faster life and reduced energy reserves and increased oxidative stress.

Is stress in the city leading to adaptive evolution?

We documented genetic differentiation of *D. magna* populations along urbanization gradients, which represent multifarious selection gradients. City ponds are warmer (Brans *et al.*, 2018a) and likely suffer more mechanical disturbances linked to management (Hassall, 2014; and personal observations) than rural ponds, and both factors might select for a faster life style (Brans & De Meester, 2018). The impact of temperature might be both direct as well as indirect, e.g. through increased feeding activity of invertebrate predators or changed biotic interactions (competition, parasitism). While urbanization potentially involves many environmental stressors, including pollution, an extensive field study on 81 ponds in the study region revealed only weak differences in nutrient concentrations (slightly lower nitrogen concentrations in urban compared to rural ponds: Engelen, 2017; Gianuca *et al.*, 2018), no significant differences in food resources (chlorophyll a concentrations quantifying phytoplankton abundances: Engelen, 2017; Gianuca *et al.*, 2018), and no significant differences between urban and rural ponds in overall metal contamination (26 metals) and pesticide concentrations (21 pesticides) between urban and rural ponds (Engelen, 2017). This suggests that differences in water quality or food resources are likely not a recurrent selection factor differentiating rural and urban ponds, although we interpret these data cautiously because these environmental data were collected during a single snapshot sampling campaign.

The genetic differences we observed between urban and rural populations most likely reflect adaptive evolution to cope with the higher temperatures and stress levels associated with anthropogenic disturbances in urban environments. Our study populations were scattered over a quite large region involving different cities and our results therefore suggest that city populations consistently behave differently from rural ones irrespective of the region from which they were collected. As we did not test for fitness differences between urban and rural populations, non-adaptive evolutionary processes can, however, not be ruled out as drivers of the observed genetic differentiation. Depending on the genetic

architecture of the traits, genetic drift might generate a repeatable signal (Santangelo *et al.*, 2018). The broad set of responsive traits (physiology, life history and heat tolerance) observed in this study and previous work (Brans *et al.*, 2017b; Brans & De Meester, 2018), and the fact that heat tolerance has a direct fitness link to temperature differences, provides further evidence for adaptive evolution as a driver of the observed genetic differentiation between urban and rural populations. Finally, the significant effect of genotype, nested within population, that we observe, indicates the presence of evolutionary potential for four out of seven traits measured.

Conclusion and future directions

We show that urban *Daphnia p*opulations differ genetically from rural ones for a suit of traits linked to energy metabolism and stress physiology. Additionally, urbanization structures the presence of an overarching pace-of-life syndrome linking life history and stress physiology. Strikingly, the direction of evolutionary divergence in mean traits when comparing urban and rural populations is different from the evolved POLS-direction within the urban populations. The latter involves a classic trade-of between fast development, coinciding with energy depletion, and higher oxidative stress levels. Possibly, the evolution of higher energy reserves and more efficient stress coping mechanisms in urban populations provides the needed energy to drive the evolution of a faster pace-of-life, which is then established via the evolved overarching POLS, but nevertheless comes at a cost of energy depletion and increased oxidative stress. Our results point to the need for more work on stress physiological responses and the mechanistic underpinning of trait covariation among and within sets of genotypes (Peiman & Robinson, 2017). Additionally, an effort should be made to include subcontinental (e.g. European) or global scale studies as these will reveal whether our observations can be generalized at larger spatial scales (Thompson *et al.*, 2016; Johnson & Munshi-South, 2017). While common garden experiments as used in the present study, involving urban and rural populations sampled across different urban cores in the same region (Flanders, Belgium), provide strong evidence for genetic differentiation in ecologically relevant traits, they are labor-intensive, which limits their application to large-scale survey studies. The capacity to detect urban evolution via genomic signatures would be a major step forward, given that patterns can then be compared across regions and taxa (Harris & Munshi-South, 2017). Finally, there is a strong need to quantify the consequences of urban evolution for ecosystem functioning and services to society, such as pollination, pest control, and top-down control of primary producers. Additionally, given the strong temperature differences observed between urban and rural ponds, urban environments may provide a window into conditions pond dwelling organisms will experience as the climate warms.

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We thank Marc Johnson, Ruth Rivkin, James Santangelo, and three anonymous reviewers for their constructive feedback and comments on the manuscript. We thank Ria Van Houdt for her contribution

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Supplementary Information "Urbanization drives genetic differentiation in physiology and structures the evolution of pace-of-life syndromes in the water flea *Daphnia magna*"

SUPPLEMENTARY INFORMATION A: FIGURES AND TABLES

Figures

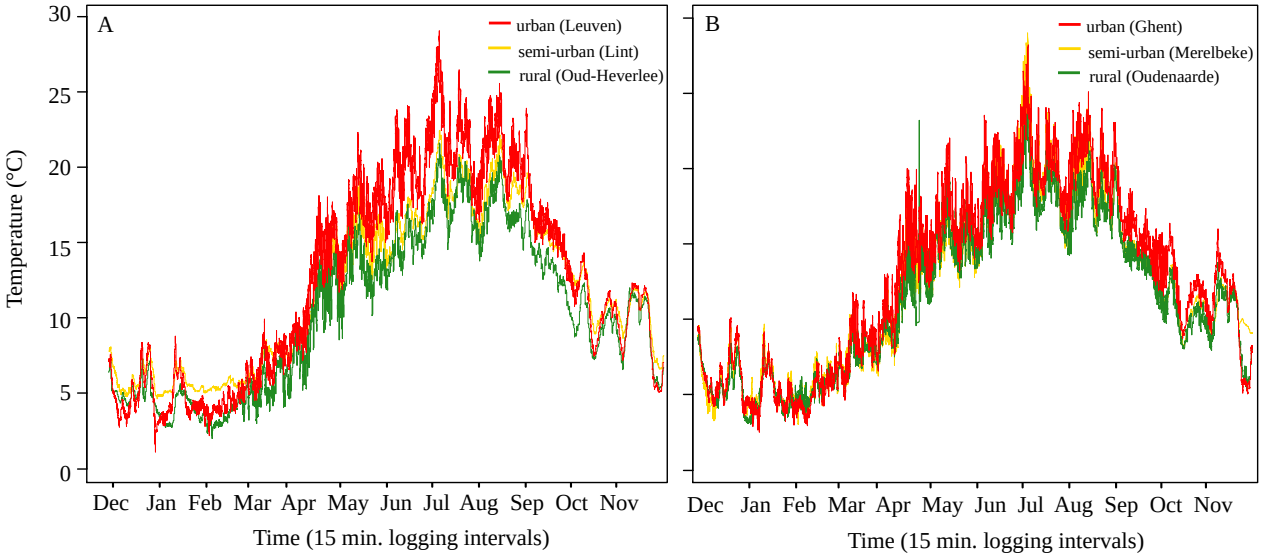

Figure S1. Temperature profiles (°C, measured at 15 min. intervals; period November 2014- November 2015) for an urban (red), semi-urban (yellow), and rural (green) pond in the regions of (A) Leuven and (B) Gent in Flanders, Belgium, in the eastern and western part of the region of Flanders, respectively. Adapted from Brans *et al.* (2018a).

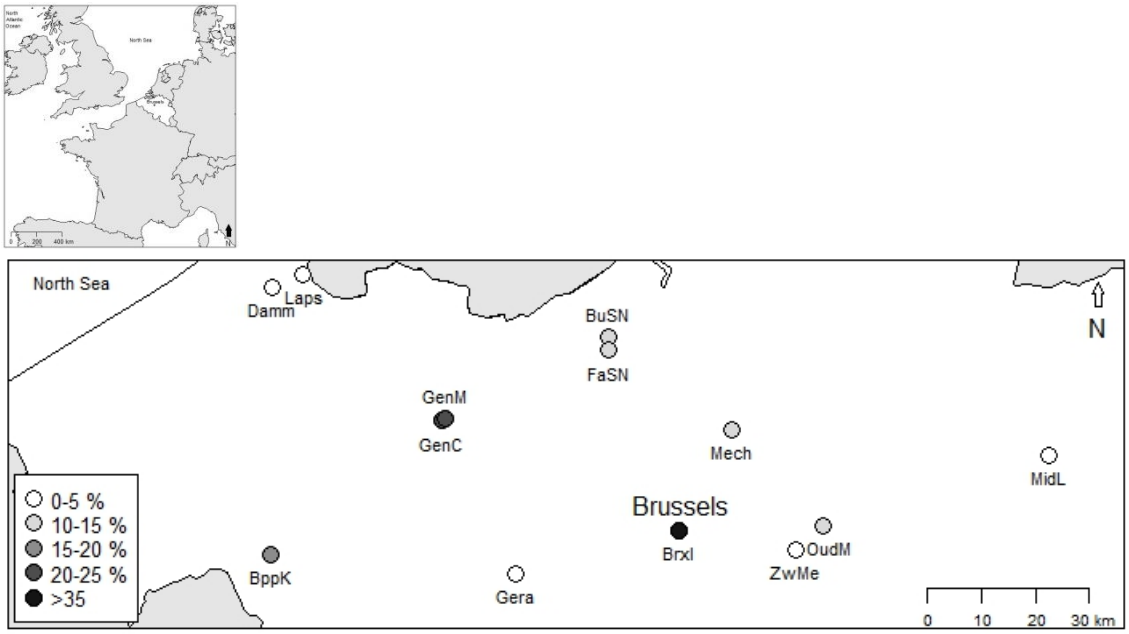

Figure S2. Sampling locations of the *D. magna* study populations in Flanders. Urbanization classes are given according to the percentage built-up area in a radius of 3200 m around each pond (< 5% BA: rural, n = 5; > 10% BA: urban, n = 8). Inset: Wider region of Europe, depicting Belgium. Adapted from Brans *et al.* (2017a,b).

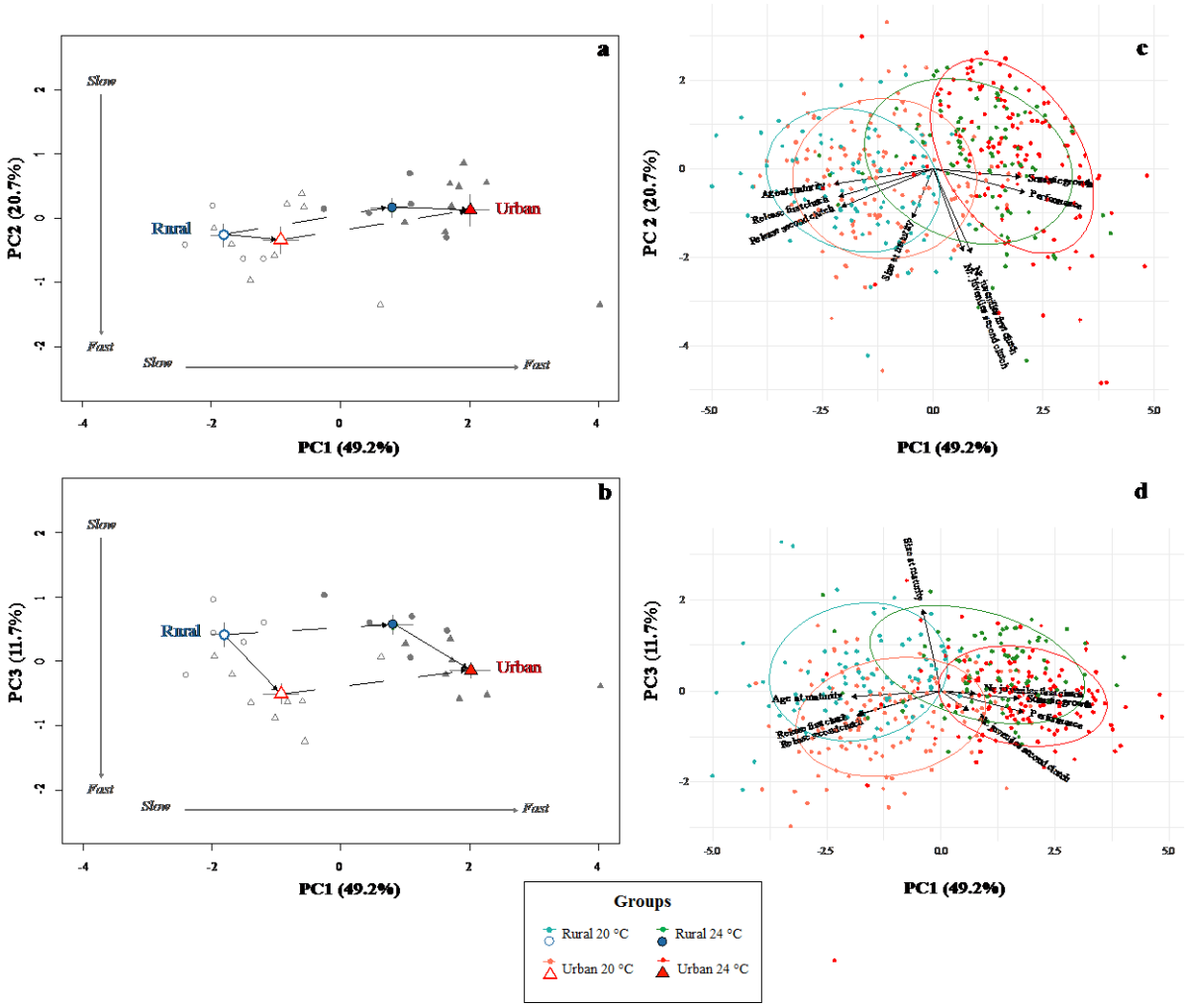

Figure S3. Multivariate life history results. Principal Component Analysis plots showing: (a,b) phenotypic trajectories (multivariate response to experimental warming for eight morphological and life-history traits; dashed lines) and genotypic trajectories (multivariate differentiation between rural and urban animals; full lines) for rural (blue circles) and urban (red triangles) populations of the water flea *Daphnia magna* reared at 20 (open symbols) and 24 °C (full symbols); population means are depicted in grey shades (white: 20 °C, gray: 24 °C), average 'urban' and 'rural' centroids are depicted in the according color (rural: blue; urban: red); (c,d) PCA plots showing all observations (n = 440) grouped according to the urbanization x temperature combinations (rural-20 °C: blue, rural-24 °C: green, urban-20 °C: orange, urban-24 °C, red). Patterns are given for PC1 and PC2 (a,c) and PC1 and PC3 (b,d). An indicative 'pace-of-life' axis along all PC axes is given in plots a and b. Adapted from Brans & De Meester (2018).

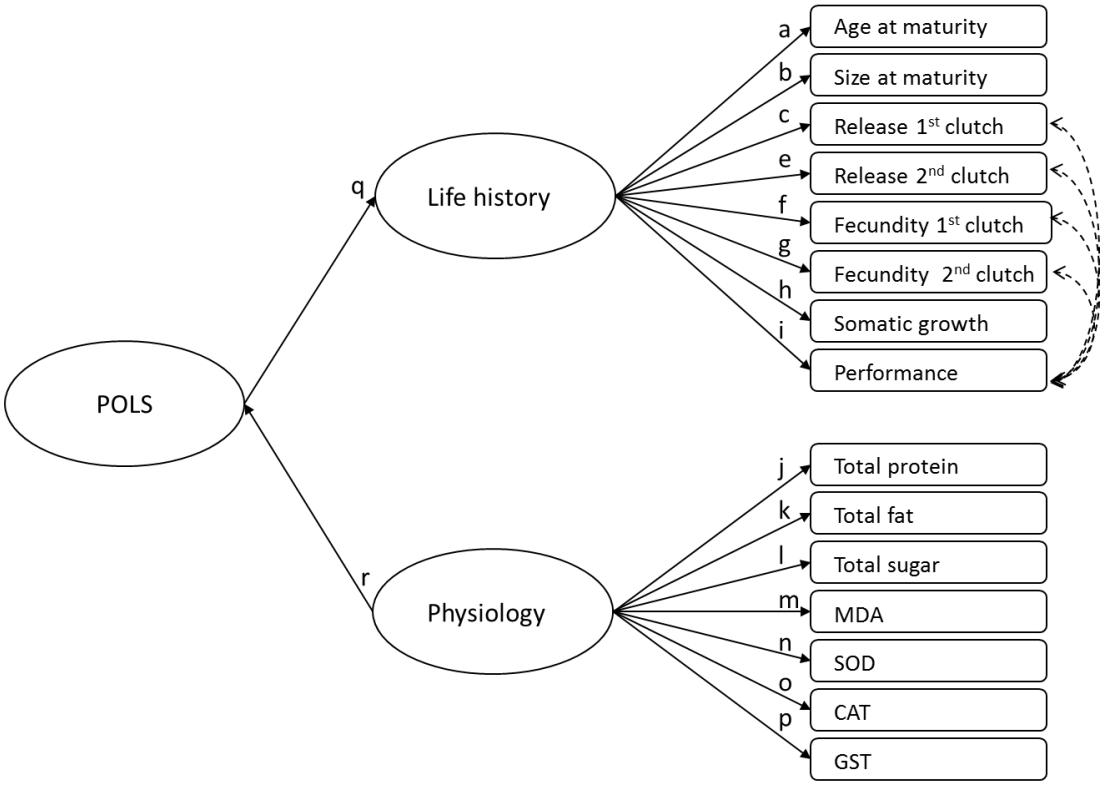

Figure S4. Latent variable structural equation model. A priori path model between life-history traits and physiology traits, potentially shaped by a life-history syndrome structure (paths a-i),a physiology syndrome sturcure (paths j-p), and an overarching syndrome structure (paths q and r) according to the pace-of-life Syndrome Hypothesis (Réale *et al.*, 2010). "Syndromes" underlying the possible trait covariation of measured variables (boxes) at each level of biological organization are modelled as 'latent', non-measured, variables (ovals). Dashed arrows are correlated errors, indicating residual covariation not related to the syndrome structure or path model.

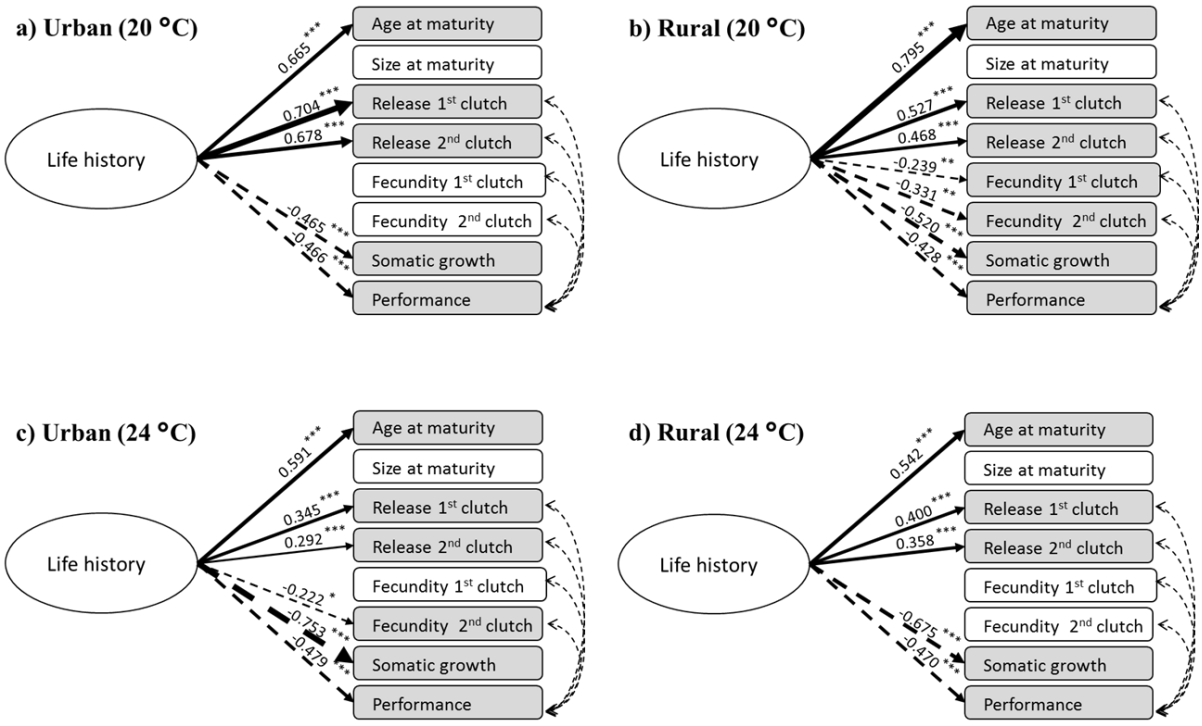

Figure. S5. Structural equation models life-history syndrome. Path models showing the covariation pattern of measured life-history traits (boxes) within an overarching life-history syndrome (modeled as latent variable, ovals) in urban (a,c) and rural (b,d) genotype sets of *Daphnia magna* when reared at 20 °C (a,b) and 24 °C (c,d). The best model supporting the underlying syndrome structure was characterized by factor loadings free across all temperature x urbanization groups (Model IV, Table S3). Only paths significantly contributing to syndrome structure (i.e. significantly different from 0) are depicted (associated traits are in grey colored boxes), and accompanied with path coefficients. Significance levels of path coefficients are depicted as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Path coefficients indicate the change (in SD units) of traits predicted to occur based on a 1 SD change in the underlying syndrome structure. Arrow thickness (dashed arrows: negative correlations, full arrows: positive correlations) is proportional to the strength of the path loading. Dashed double-headed arrows indicate correlated errors.

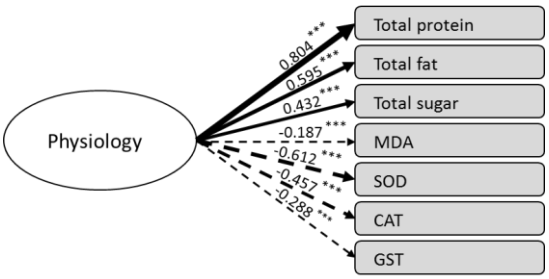

Figure S6. Structural equation model physiology syndrome. Path model showing the covariation pattern of measured stress physiology traits (boxes) within an overarching physiology syndrome (modeled as latent variable, ovals). The best model supporting the underlying syndrome structure was characterized by factor loadings constraint across all temperature x urbanization groups (Model I, Table S3). Only paths significantly contributing to syndrome structure (i.e. significantly different from 0) are depicted (associated traits are in grey colored boxes), and accompanied with path coefficients. Significance levels of path coefficients are depicted as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Path coefficients indicate the change (in SD units) of traits predicted to occur based on a 1 SD change in the underlying syndrome structure. Arrow thickness (dashed arrows: negative correlations, full arrows: positive correlations) is proportional to the strength of the path loading.

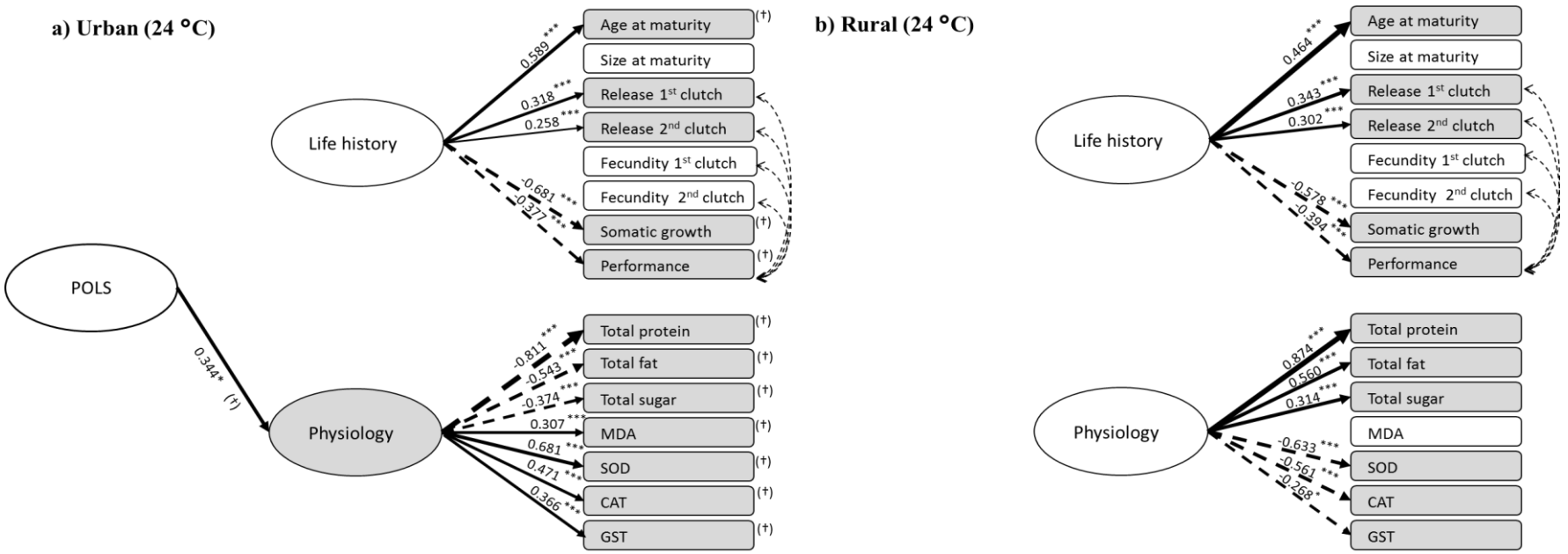

Figure. S7. Structural equation models. Path models showing the covariation pattern of life-history and physiological traits (i.e. measured traits, boxes) within a life-history, physiology, and overarching pace-of-life syndrome (POLS) (all modeled as latent variable, ovals) in a) urban and b) rural genotype sets of *Daphnia magna* when reared at 24 °C. Only paths significantly contributing to syndrome structure (i.e. significantly different from 0) are depicted (associated traits are in grey colored boxes), and accompanied with path coefficients. Significance levels of path coefficients are depicted as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Path coefficients indicate the change (in SD units) of traits predicted to occur based on a 1 SD change in the underlying syndrome structure. Arrow thickness (dashed arrows: negative correlations, full arrows: positive correlations) is proportional to the strength of the path loading. Path loadings significantly differing between urban and rural genotype sets (either because of their significant contribution to a syndrome in one group, but not in the other, or because of a significant difference in direction or size of path loadings that significantly contribute to syndrome structures in both groups) are indicated with † ($p < 0.05$ after Bonferroni correction). Dashed double-headed arrows indicate correlated errors.

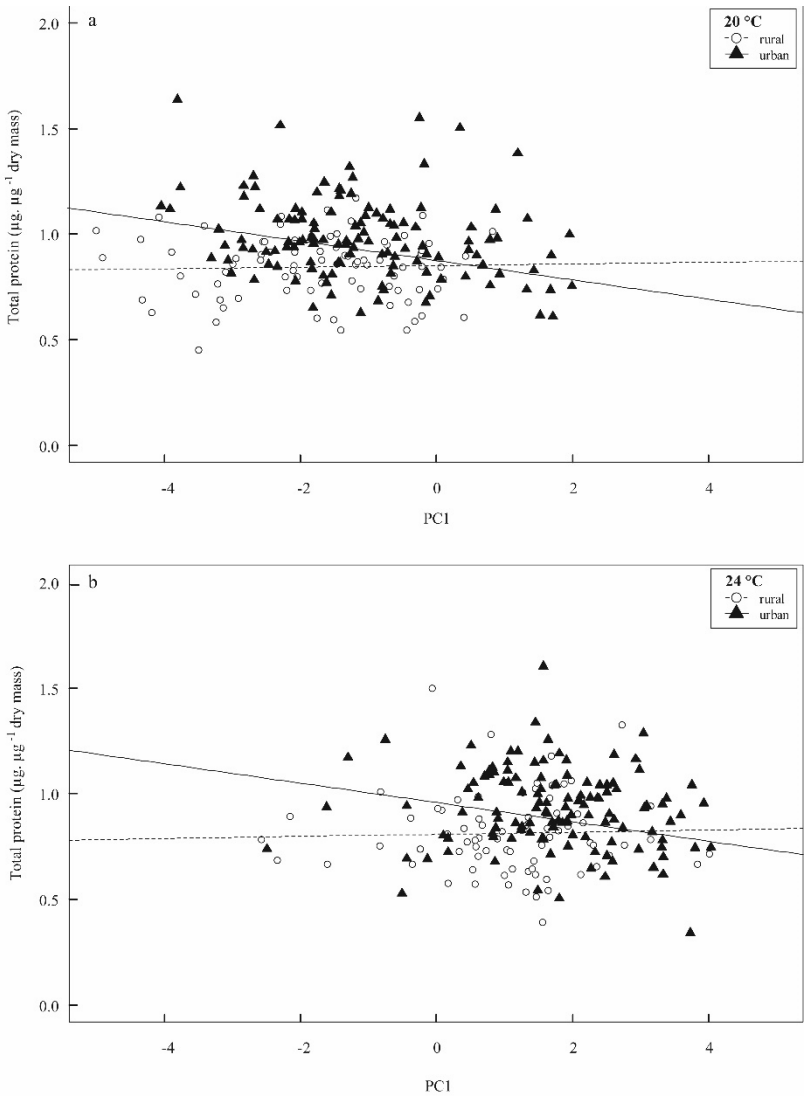

Figure. S8. Total protein content regressed against pace-of-life (life history). Total protein content plotted against PC1 scores from a multivariate analyses including all measured life-history traits in urban (blak triangels) and rural (white circles) *D. magna* genotypes. Regression lines are computed from the statistical output from a linear mixed-effect model, including the effects of PC1, urbanization, temperature, and all possible interactions between the latter on total protein content. A significant PC1 x urbanization interaction effect ($p = 0.008$) was observed, indicating slopes differed in urban (negative correlation) and rural (no correlation) genotype sets. For the sake of clarity, we here plotted data obtained from both temperature treatments (20 °C and 24 °C) separately.

Tables

Table S1. *Daphnia magna* populations. Locations of the *D. magna* study populations including the percentage built-up area (BA) in a radius of 3200 m around each pond, the according urbanization categorization, and the number of experimental lines and clones (i.e. multi-locus genotypes, MLGs) used in the experiment. Adapted from Brans *et al.* (2017b).

Population		**Location**		**Urbanization**		**Exp. lines**
Pond	Code	Latitude (N)	Longitude (E)	%BA	Cat.	(MLGs)
Brussel	*Brxl*	50.855464	4.347334	40.440	urban	4(4)
Damme	*Damm*	51.26207	3.276039	1.126	rural	6(6)
Gent (Citadelpark)	*GenC*	51.03890	3.723744	24.242	urban	6(6)
Gent (Muinkpark)	*GenM*	51.04251	3.731611	25.736	urban	6(5)
Geraardsbergen	*Gera*	50.78420	3.915978	4.382	rural	6(3)
Kortrijk (Blauwe Poort)	*BppK*	50.81624	3.271563	15.356	urban	6(6)
Lapscheure	*Laps*	51.28253	3.355467	0.637	rural	6(5)
Leuven (Oude-Meren)	*OudM*	50.86328	4.723935	13.188	urban	6(5)
Mechelen (Kruidtuin)	*Mech*	51.02402	4.484039	14.125	urban	6(2)
Meerdaal (Zoete Waters)	*ZwMe*	50.82274	4.653691	2.017	rural	6(6)
Midden-Limburg (BKN1)	*MidL*	50.98233	5.317858	3.721	rural	6(5)
Sint-Niklaas (Bufferbekken)	*BuSN*	51.17808	4.161051	12.479	urban	6(4)
Sint-Niklaas (Fabiola Park)	*FaSN*	51.15623	4.159557	14.995	urban	6(5)

Table S2. Multi-group structural equation modeling (SEM). Alternative path models to test for i) overall covariation in life-history and physiological traits according to a life-history, physiology, and overarching pace-of-life syndrome, ii) differences in model structure across urbanization level and/or temperature treatment for the life-history, physiology, and overarching pace-of-life syndrome. Each model is a multi-group SEM with all combinations of urbanization x temperature treatment as grouping structure. Each alternative SEM is characterized by a different set of constrained factor loadings (all, temperature, urbanization level, none; equal factor loadings are indicated by an identical character). For more information on the interpretation on model structures we refer to Supplementary Information B: Material and Methods.

		Urban		**Rural**	
Model		**20 °C**	**24 °C**	**20 °C**	**24 °C**
I	All loadings constrained equal across treatments and urbanization level	A	A	A	A
II	Loadings constrained across temperature treatment (*free across urbanization level)*	A	A	B	B
III	Loadings constrained across urbanization level *(free across temperature treatment)*	A	B	A	B
IV	All loadings free	A	B	C	D

Table S3. Multi-group SEM results. Four models of covariation of life-history and physiological traits along separate life-history and physiological syndromes (A, B) and an overarching pace-of-life syndrome (C) were evaluated with factor loadings (I) constrained across both urbanization level and temperature treatment groups, (II) constrained across temperatures (free across urbanization levels), (III) constrained across urbanization levels (free across temperatures), (IV) free across all groups. Model AICs are given together with ΔAIC (based on the best fitting model's AIC score), together with model weights (*W*) and evidence ratios (E.R.). The optimal model for each syndrome structure is given in bold. Competing models (ΔAIC < 2) are given in italics.

	Model	**Factor levels constrained across**	**AIC**	**ΔAIC**	***W***	**E.R.**
A) Life-history SEMs	I	Temp. x Urb.	5739.60	134.38	0.00	$7.35\ E^{+39}$
	II	Temperature	5744.18	188.168	0.00	$7.25E^{+40}$
	III	Urbanization	5579.97	23.96	0.00	159452.28
	IV	**All free**	**5556.01**	**0**	**0.99**	**1**
B) Physiological SEMs	***I***	**Temp. x Urb.**	***7302.31***	***0***	***0.59***	***1***
	II	Temperature	*7303.99*	*1.68*	*0.25*	*2.32*
	III	Urbanization	7304.99	2.67	0.15	3.81
	IV	All free	7312.18	9.86	0.004	139
C) Full POLS SEMs	I	Temp. x Urb.	12602.85	131.7	0.00	$4.09E^{+28}$
	II	Temperature	12616.73	145.64	0.00	$4.22E^{+31}$
	III	Urbanization	12477.47	6.38	0.04	24.29
	IV	**All free**	**12471.09**	**0**	**1**	**1**

SUPPLEMENTARY INFORMATION B: MATERIAL AND METHODS

Quantification of response variables

Energy storage compounds

The pooled sample of animals per replicate of a clonal line-by-treatment combination was homogenized with a pestle in PBS buffer (Phosphate-Buffered Saline, 50 mM, pH: 7.4, 50 µl/animal) and centrifuged for 8 min at 13000 rpm and 4°C. All response variables were measured on the resulting supernatant using a Tecan plate reader, set at the appropriate wave length.

After a 5 minute incubation (25 °C) with the Bradford Reagens (Sigma-Aldrich B6916, Bradford, 1976), total protein content was measured (at 595 nm) in quadruplicate and the means per pooled sample were used for the statistical analyses. Total protein concentration was expressed in µg per µg dry mass of the set of animals that were homogenized.

To quantity total fat content we followed the protocol of Bligh & Dyer (1959). H_2SO_4 (100%) was added to the homogenate, which was left to incubate at 150 °C (20 min.). After cooling down, total fat content was assessed in technical triplicate by measuring absorbance at 340 nm. Fat concentration was calculated based on a standard curve of glyceryl tripalmitate and expressed as µg per µg dry mass.

Total sugar content (glucose + glycogen) was measured following the protocol described in Stoks *et al.* (2006) using a glucose oxidase kit (MAK097-1KT, Sigma Aldrich). We first added an amyloglucosidase solution (Sigma A7420) to the homogenate, which was then left to incubate at 37 °C for (30 min.). In a next step a glucose reagent was added to the same sample and left to incubate at 30 °C for 20 min. Absorbance was measured at 340 nm. To distinguish glycogen from glucose components, the second step was repeated on a new subsample of the homogenate, after which the resulting concentration was subtracted from the first measurement (i.e. (glucose + glycogen) – glucose). Total sugars (glucose + glycogen) was used as response variable in the statistical analysis.

Antioxidant defense

The activity of superoxide dismutase (SOD), catalase (CAT), two known key antioxidant enzymes in insects and invertebrates (Korsloot *et al.*, 2004), and gluthation-S-transferase (GST), a secondary anti-oxidative enzyme which protects against oxidizing and toxic substances by detoxifying ROS-damaged cellular components was measured as an estimate of antioxidant defense.

SOD activity was quantified using the protocol of De Block & Stoks (2008), based on the SOD assay kit WST (Fluka, Buchs, Austria). WST working solution was mixed with enzyme working solution and the homogenate of the sample. After incubation (20 min. at 37 °C), absorbance was

measured at 450 nm. SOD acitivity is defined as the percentage inhibition of the reduction reaction of WST-1 with superoxide anion and is standardized per µg protein.

CAT activity was measured following De Block & Stoks (2008); after a homogenate dilution with PBS (16 times), 100 µl of 20 mM H_2O_2 was added (to 20 µl of the diluted sample and 80 µl PBS [100 mM, pH 7.4]). CAT activity was then quantified as the degradation rate of H_2O_2 with absorbance being measured (duplicate measurements) every 30 seconds (12 readings) at 240 nm. Using the slope of the linear part of the reaction curve, CAT activity was calculated as the amount of H_2O_2 decomposition per minute and per µg protein.

GST activity was quantified based on the protocol of McLoughlin *et al.* (2000). 5 µl of sample was added to 195 µl of substrate solution (2 mM glutathione (GSH) solution, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution). The filled 96 well microtiter plate (suited for UV-spectrum) was pre-incubated for three minutes at 30 °C, after which absorbance was read in duplicate at 340 nm every 30 seconds for 10 min at 30 °C. GST activity was calculated based on the slope of the linear part of the reaction plot, as the amount of GS-DNB formed per minute and per µg protein.

Oxidative damage

A HPLC-based TBARS essay was conducted to measure malondialdehyde (MDA) levels (Grotto *et al.*, 2009), a byproduct of lipid peroxidation. Sample preparation was done as in Debecker (2017): 50 µl of homogenate sample was mixed with an equal amount of thiobarbituric acid (TBA; 0.4% [40 mg TBA in 10 ml 0.2 M HCl]). After a 1-hour incubation at 90 °C, 165 µl butanol (100%) was added to the sample, which was mixed and centrifuged (3 min., 4 °C, 13000 rpm). 50 µl of this supernatant was then injected in an HPLC/UV-Vis system with a 10 µl loop on a C18 column (250 x 4.6 x 5 µm; chromatogram measurements at 535 nm). MDA concentrations were expressed as nmol MDA per µg dry mass. Total fat content was used as a covariate in the statistical analysis.

Statistical analysis

MANOVA and linear mixed-effect models

To test for differentiation in physiological traits between urban and rural *Daphnia* (categorical), and between animals reared at 20 °C and 24 °C (categorical), we first performed a MANOVA, followed by separate linear mixed-effect models on all response variables scaled to a mean of 0 and standard deviation of 1. Fixed factors were urbanization, temperature treatment, and their interaction term. A random error term for 'Clone' (i.e. genotypic identity of each line; see Table S1 of Supplementary Information A) nested in population was added. Pillai's trace test statistic was extracted using the 'car' package (Fox & Weisberg, 2010; R Development Core Team, 2016). Next, we ran separate linear mixed-effect models ('lme4' package, Bates *et al.*, 2014; R Development Core Team, 2016) for each

of the seven response traits (same model structure; fixed effects: urbanization level, temperature treatment, and urbanization x temperature treatment; random effects: clone, nested in population, and population, nested in urbanization class). F-test statistics were computed using the 'car' package. F-tests were fitted with the restricted maximum likelihood ratio; degrees of freedom were corrected using the Kenward-Roger approximation. To assess a possible effect of clonal identity (implying genetic variation for a certain trait within populations and thus evolutionary potential), we refitted each linear mixed-effect model without this random factor and performed a model comparison (likelihood ratio test with χ^2 distribution; p-values were halved according to 1-tailed testing).

Assumptions of normality and homogeneity of variances were checked for each model visually (by plotting model residual histograms, QQ-plots, and residual vs. fitted values) and tested for by performing Shapiro-Wilk's test of normality on model residuals and Levene's test for homogeneity of variance on each variable (using either treatment or urbanization as a grouping factor). All model assumptions were met except for MDA, which was log-transformed to better meet residual normality.

Structural equation modelling

First we assessed trait covariation patterns in life-history traits and physiology traits separately. In a last SEM analysis we assessed the possible presence of an overarching POLS by integrating both life-history and physiological traits. For each SEM (Grace, 2006), we compared four alternative models with different combinations of factor loadings (i.e. path coefficients) constrained to be equal vs. freely varying across all urbanization x temperature groups (i.e. urban-20 °C, rural-20 °C, urban-24 °C, rural-24 °C; Table S2, Supplementary Information A): (I) loadings were constrained to be equal across temperature treatment and urbanization level, (II) loadings were constrained to be equal across temperature treatments only, (III) loadings were constrained to be equal across urbanization level only, and (IV) loadings are all free (Table S2). For example, if a model with loadings constraint across temperature treatments (model II) is regarded the most appropriate model explaining the trait covariation patterns, it in essence indicates trait covariation patterns are the same in animals reared at 20 or 24 °C, but are different for urban vs. rural populations. This thus means urbanization (i.e. evolutionary processes) shapes trait covariation patterns, given that rural and urban populations are characterized by different factor loadings and consequently different trait covariation patterns. If a model with loadings constraint across urbanization level is the most appropriate (model III), it indicates trait covariation patterns are similar between urban and rural populations, but different when comparing animals reared at 20 °C vs. 24 °C. In case model selection leads to model IV being the best fit, indicated by factor loadings freely varying across all urbanization x temperature treatment groups, trait covariation patterns differ between urban animals reared at 20 °C, urban animals reared at 24 °C, rural animals reared at 20 °C, and rural animals reared at 24 °C. In the last case, both urban background

(evolution/genetic differentiation) and temperature treatment (plasticity to warming), impact trait covariation patterns.

Based on AIC scores the most appropriate model was chosen. In case $\Delta AIC < 2$, we assessed model weights (*W*) and evidence ratios (E.R.) (Burnham & Anderson, 2002; Wagenmakers & Farrel, 2004) to validate model choice. *W* characterizes the probability the evaluated model is the most appropriate, given the set of models tested. E.R. is calculated by dividing the model weight of the best model (lowest AIC) by the weight of the evaluated model and thus represents the likelihood of the best model compared to the evaluated model.

In case models II, III, or IV were selected as best model (i.e. a non-fully restricted model), group differences of interest were compared by comparing factor loadings (significance, direction, and size) using the method of Zar (1999). Path coefficients of two contrasted groups were subtracted from each other and divided by the pooled standard error, after which values were evaluated as t-values and significances were assessed. P values were corrected for multiple testing using Bonferroni corrections (paths significantly differing - $p < 0.05$ - between groups of interest will be indicated with a †).

SUPPLEMENTARY INFORMATION C: RESULTS

Structural equation modelling: exploring the Pace-of-life Syndrome Hypothesis

We here present more detailed results on the separate life-history and physiology SEMs

Life-history SEMs – life-history trait covariation patterns

The path analysis on life-history traits revealed a syndrome structure with underlying life-history trait covariation differing between all tested groups (model IV, Table S3, factor loadings all free; Fig. S5, Supplementary Information A). The difference in syndrome structure all four groups was related to fecundity (first and second clutch juveniles), which was significantly contributing to the life-history syndrome (i.e. significantly different from 0) in rural animals reared at 20 °C, but not in urban animals reared at 20 °C ($p < 0.001$). Rural animals exhibiting a longer time to maturation and release of the first clutch have a lower number of juveniles in the first and second clutch. This association is not present in urban animals. At 24 °C (Fig. S5), second clutch fecundity was negatively associated with age at maturation timing and at release of the first and second clutch in urban animals ($p < 0.001$).

Physiology SEMs – Physiology trait covariation patterns

The best model explaining a structuring syndrome at the physiological level was model I (Table S3, all factor loadings constrained to be equal: Fig. S6, Supplementary Information A), indicating a consistent trait covariation across all four urbanization x temperature groups. There is a consistent pattern, with animals with higher concentrations of fat, proteins, and sugars having lower levels of MDA and a reduced activity of SOD, CAT, and GST (all factor loadings significantly different from 0). Although this model could not be distinguished from model II (Table S3, $\Delta < 2$; factor loadings constrained across temperature treatment, but free across urbanization level), model weights and evidence ratios show the best model (i.e. model I) is twice as likely to occur compared to model II.

Part 3: Eco-evolutionary dynamics in the city

Chapter 5

Eco-evolutionary dynamics in urbanized landscapes: evolution, species sorting and the change in zooplankton body size along urbanization gradients

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To spare some trees, the supplementary information sections of this paper are not included here. They can be found online:
http://rstb.royalsocietypublishing.org/content/royptb/suppl/2016/11/28/rstb.2016.0030.DC1/rstb20160030_si_001.pdf

http://rstb.royalsocietypublishing.org/content/royptb/suppl/2016/11/28/rstb.2016.0030.DC1/rstb20160030_si_002.pdf).

Abstract

Urbanization causes both changes in community composition and evolutionary responses, but most studies focus on these responses in isolation. We performed an integrated analysis assessing the relative contribution of intra- and interspecific trait turnover to the observed change in zooplankton community body size in 83 cladoceran communities along urbanization gradients quantified at seven spatial scales (50–3200 m radii). We also performed a quantitative genetic analysis on 12 *Daphnia magna* populations along the same urbanization gradient. Body size in zooplankton communities generally declined with increasing urbanization, but the opposite was observed for communities dominated by large species. The contribution of intraspecific trait variation to community body size turnover with urbanization strongly varied with the spatial scale considered, and was highest for communities dominated by large cladoceran species and at intermediate spatial scales. Genotypic size at maturity was smaller for urban than for rural *D. magna* populations and for animals cultured at 24 °C compared with 20 °C. While local genetic adaptation likely contributed to the persistence of *D. magna* in the urban heat islands, buffering for the phenotypic shift to larger body sizes with increasing urbanization, community body size turnover was mainly driven by non-genetic intraspecific trait change.

INTRODUCTION

In the past decade, a growing number of empirical studies have provided evidence that micro-evolutionary change can occur at the same temporal scale as ecological change and, therefore, can influence ecological dynamics (Hendry, 2016). The importance of eco-evolutionary feedbacks in biological responses to changing environments caused by, for example, interactions with antagonists (e.g. Becks *et al.*, 2012), or by human disturbance, such as agriculture (Loeuille *et al.*, 2013) and climate change (e.g. Van Doorslaer *et al.*, 2009) led to the development of eco-evolutionary partitioning metrics. These metrics quantify the relative contribution of evolutionary and ecological processes to trait change and have revealed that effect sizes of evolutionary change can be equal to or even higher than those of ecological change (Hairston *et al.*, 2005; Becks *et al.*, 2012; Pantel *et al.*, 2015). Most eco-evolutionary studies, however, focus on population level dynamics (e.g. Olsen *et al.*, 2004; Phillips & Shine, 2004) and only a few studies quantify the influence of evolutionary change on community structure (Bassar *et al.*, 2010; Pantel *et al.*, 2015; Matthews *et al.*, 2016) beyond specific predator–prey or host–parasite interactions (e.g. Becks *et al.*, 2012). In addition, most community-level studies focus on intraspecific phenotypic variation (Violle *et al.*, 2012; Lajoie & Vellend, 2015) rather than also quantifying the genotypic component to trait change.

Urbanization has been shown to elicit both ecological and evolutionary responses in different organism groups, impacting biodiversity patterns and ecosystem functioning (Shochat *et al.*, 2006; McKinney, 2008). At the community level, a significant decrease in mammal, reptile, amphibian, invertebrate and plant species richness has been observed in highly urbanized areas (McKinney, 2008). At the level of individual populations, a broad range of adaptive behavioral, morphological and physiological responses of urban populations have been reported (reviewed in Donihue & Lambert, 2015). For example, the plant species *Crepis sancta* shows a reduced settling success of dispersing seed types and a significant increase in non-dispersing seed types in urban areas associated with increased habitat fragmentation (Cheptou *et al.*, 2008; Cheptou & Dubois, 2016). Tüzün *et al.* (2015) recently reported altered behavioural responses to a pesticide in replicated urban populations of the damselfly *Coenagrion puella*, suggesting local adaptation to higher pesticide concentrations in urban environments. There is a strong need to integrate evolutionary and ecological responses to urbanization (Alberti, 2015). This integration should not only include an assessment of the importance of intraspecific trait variation (ITV; i.e. within species trait variation among environments that is a result of either phenotypic plasticity, ontogenetic shifts or genetic adaptation) relative to species trait turnover (SPT; i.e. trait change caused by the change in relative abundances of species; it thus reflects trait change caused by interspecific trait variation) along the urbanization gradient, but should also clarify how much of ITV is the result of genetic responses compared with environmental modulation through phenotypic plasticity or ontogenetic change. In addition, there is a need to account for scale effects of urbanization.

Because human-dominated landscapes generate intense and novel selection pressures on very short spatial and temporal scales, urban areas provide a powerful system to monitor evolutionary trait change and the reciprocal interplay with ecological dynamics across taxa (Alberti, 2015; Cheptou *et al.*, 2016; Hendry *et al.*, 2016; Lancaster *et al.*, 2016). Cities differ from rural areas in hydrology, atmospheric chemistry, microclimate, nutrient concentrations, land use and vegetation cover (Alberti *et al.*, 2003; Kowarik, 2011; Gilbert, 2012). Urban areas are characterized by higher eutrophication, soil and water contamination by organic pollutants and heavy metals, noise and light pollution, and usually have a low overall habitat quality and connectivity (Kaye *et al.*, 2006; Grimm *et al.*, 2008a,b). One of the striking environmental changes linked to urbanization is the significant increase in ambient surface temperature compared with rural surroundings ('urban heat island effect', UHI; Oke, 1973; Arnfield, 2003). These have also been studied in detail in the northern provinces of Belgium (Flanders) during the past decade (De Ridder *et al.*, 2015). The average difference in summer mean surface temperature between cities and rural areas in Flanders ranged between ca. 1.4 °C and ca. 4.5 °C (with extreme observations up to 8 °C differences in summer day temperature) and was positively correlated with the level of impervious surfaces.

As a result of the increased average and extreme temperatures, we expect that urban dwellers will show a reduced body size in many organism groups to better optimize physiological oxygen transport and respiration processes ('temperature–size rule'; Atkinson, 1994). Body size is a key trait in freshwater zooplankton, as it is a strong determinant of SPT along environmental gradients (e.g. fish predation pressure; Brooks & Dodson, 1965; Kerfoot & Sih, 1987) as well as of performance in competition and top-down control of algae (Tessier *et al.*, 2000; Gianuca *et al.*, 2016). The presence of large zooplankton species, such as the water flea *Daphnia magna*, can prevent shifts from the clear-water to the turbid stable state in ponds and shallow lakes (Scheffer *et al.*, 1993). Oxygen limitation can, however, be severe in aquatic systems (e.g. Horne *et al.*, 2015). It is currently not known to what extent urban zooplankton populations show a reduced body size compared with rural populations, to what extent patterns in body size variation run parallel for intra- and interspecific trait turnover, and to what degree changes in body size along urbanization gradients also have a genetic component.

We here performed a partitioning analysis to better understand the underlying processes leading to variation in body size in zooplankton communities along strong urbanization gradients using the variation partitioning method developed by Lepš *et al.* (2011) and adapted by Kichenin *et al.* (2013) and Lajoie & Vellend (2015). This method enables us to assess the relative importance of ITV versus SPT to community body size turnover along the urbanization gradient. Moreover, we adapted this method to integrate both genetic and non-genetic ITV, which constitutes a novel approach towards disentangling genotypic trait variation (GTV) from other ITV components and SPT. To do so, we combined zooplankton community field data and common garden experimental data on one focal species, *D. magna*. Our overall aim was to disentangle the underlying mechanisms shaping community

trait–environment tracking along urbanization gradients. Environmental filtering is expected to lead to community body size turnover along the urbanization gradient, and we here want to assess to what extent this is caused by intra- versus interspecific trait variation. Our specific hypotheses are (i) that ITV contributes significantly to the observed community trait turnover, (ii) that the patterns in intra- and interspecific body size variation covary along the urbanization gradient, and (iii) that evolutionary processes (i.e. genetic change) contribute significantly to intraspecific and overall community trait turnover in this system. Given the importance of body size for top-down control of algae, community body size turnover with increasing urbanization resulting in an increased dominance of small zooplankton species or an evolutionary reduction in body size in dominant species could negatively affect community grazing efficiency and thereby water quality of urban ponds.

MATERIAL AND METHODS

A detailed description of Material and Methods can be found in Supplementary Information A.

Data acquisition

Habitat selection

A total of 84 permanent shallow ponds were sampled in Flanders (Belgium; latitude 51°00′ 00″ N, longitude 4° 30′00″ E, period May–July 2013; sampling locations are given in Supplementary Information A, Fig. SA1) involving gradients in urbanization along the cities of Antwerp, Brussels, Ghent and Leuven. Urbanization, assessed as percentage built-up area in 200 × 200 m and 3 × 3 km plots (%BA, i.e. surface area taken by buildings, houses and industrial infrastructure, excluding roads and parking lots) is considered high from 10% BA upwards. Ponds represented approximately equal number of estimated low (rural), intermediate and high urbanized ponds at both spatial scales. %BA was later quantified more precisely at seven separate radii around each pond (50, 100, 200, 400, 800, 1600 and 3200 m), allowing possible scale-dependent effects (e.g. a local ‘park cooling’ effect versus the more regional ‘urban heat island effect’) to be disentangled. After sampling, loggers were installed in two urban and two rural ponds, to monitor water temperature on a daily basis throughout the year (2013–2014).

Zooplankton community composition and intraspecific trait variation for body size

From each pond, a 20–40 l zooplankton sample (filtered over 64 µm) was taken using a tube sampler. At least 300 individuals were identified in each sample (Amoros, 1984; Flössner, 2000) of which body size was measured on 15 individuals of each species present in the sample. Densities were expressed as individuals per litre. For the focal species *D. magna*, an additional set of 15 adults was measured to calculate the average adult body size. A list of species present across all ponds and their mean body size

are given in Supplementary Information A, Table SA1; the number of species in each pond is given in Supplementary Information, Table SA2.

Genetic variation and phenotypic plasticity in body size in Daphnia magna

We exposed 12 *D. magna* populations inhabiting ponds along the urbanization gradient (%BA at a radius of 3200 m) to 20 °C and 24 °C in a common garden experiment to mimic the temperature gradient along the urbanization gradient (with 20 °C reflecting the average July maximum day temperature in rural ponds observed over a two-week period; see Supplementary Information, Table SA3, Fig. SA2). A total of 432 lines (12 populations × 6 lineages × 2 temperatures × 3 replicates) were grown in the laboratory under standardized laboratory conditions (water baths at 20 ± 0.6 °C, 14:10 L:D photoperiod, dechlorinated tap water, fed 1×10^5 cells ml^{-1} of the green algae *Acutodesmus obliquus* (strain number: CCAP 276/3A, formerly known as *Scenedesmus obliquus*) daily, and 80% water refreshment every other day) to obviate interference from the source habitat conditions through (grand) maternal effects. Twelve neonates (less than 24 h old, second to fourth clutch) were transferred to 500 ml jars placed in temperature-controlled water baths (20 ± 0.6 °C or 24 ± 0.4 °C), with the same photoperiod, feeding and medium refreshment regimes as described above. Size at maturity was scored at the stage of first adult instar (four individuals per cohort). We checked genotypic uniqueness of all lineages by screening variation at 27 microsatellite markers (Jansen *et al.*, 2011b). Lineages identical at all loci were considered to belong to the same clone and their data were pooled. Pond location, urbanization level and the number of clones used in our experiment are given in Supplementary Information A, Table SA4.

Statistical analysis

Data

Exploratory analyses indicated different patterns of the cladoceran community body size change depending on whether the communities were dominated by small- or large-bodied taxa (see Results). We divided the data into two subsets. The large-species dominated community subset (n = 34, large species having an average body size > 1 mm, see e.g. Brooks & Dodson, 1965; species included: *D. magna*, *D. obtusa*, *D. pulex* and the chydorid *Eurycercus lamellatus*), involving communities having more than 5% of large species in terms of abundances (a 5% cut-off value is inspired by the fact that their body size is large, so that in terms of biomass their contribution is more substantial), and the small-species dominated community subset (n = 50; all other ponds). All analyses on communities presented below were done on all three datasets (i.e. the full dataset and the two subsets). For the analyses involving genetic variation in *D. magna*, only the large-species subset was used as community dataset. Data analyses were conducted with the R software v. 3.2.3 for Windows (R Development Core Team, 2015). Outliers and influential data points were detected using the Cook's distance, the outlier test

function ('car' package, Fox & Weisberg, 2010; R development core team, 2015), and visual screening by plotting the model residuals versus leverage and plotting all the data points. The justification for outlier removal as well as the results of analyses without outlier removal are described in the Supplementary Information B.

Quantifying the relative importance of intraspecific trait variation and interspecific trait turnover on the change in cladoceran community body size

We calculated the local community body size for a community *j* first as the abundance-weighted average of the local body sizes of all species present in the local community (i.e. $\bar{z}_L^j = \sum_i q_{ij} z_{ij}$ with q_{ij} the relative abundance and z_{ij} the local average body size value of species *i* of community *j*). Second, we calculated it as the abundance-weighted average using the metacommunity-wide average body size of all species (i.e. $\bar{z}_{MW}^j = \sum_i q_{ij} \bar{z}_i$ with $\bar{z}_i$ the average body size of a species across all communities where it is present; with q_{ij} the relative abundance of a species *i* of community *j* and the metacommunity-wide average body size value of species *i* across the communities where it is present).

We partitioned the variation explained by ITV and SPT to the total trait variation along the urbanization gradients using the method described by Lajoie & Vellend (2015) (Fig. 1). We built three regression models. In the SPT + ITV model, the abundance-weighted average community body sizes using the local species body size distributions for each community was regressed against percentage built-up area, reflecting the effect of both ITV of each species and species turnover along the gradient. In the SPT model, the abundance-weighted average community body sizes using the metacommunity-wide species body sizes were regressed against percentage built-up area, accounting for the changes in the relative abundances of species and species replacements along the gradient. Determining the effect of ITV on community trait turnover along the gradient was then done by subtracting the SPT model from the SPT + ITV model as:

$$\bar{z}_L^j - \bar{z}_{MW}^j = \sum_i q_{ij}(z_{ij} - \bar{z}_i)$$

We evaluated all models at all seven spatial scales and for the three different sets of communities (i.e. the full dataset and the two subsets). Based on the Akaike information criterion (AIC), we log-transformed percentage built-up area for all models. To better meet the assumption of normality for the regression analyses, the abundance-weighted average community body sizes were also log-transformed. One extreme outlier was detected in the ITV model and removed from the full dataset (n = 83; see Supplementary Information A, Fig. SA3). For the subset of communities dominated by large species two outliers were removed (n = 32, see Supplementary Information A, Fig. SA3), and for the subset of communities dominated by small species one outlier was removed (n = 49; see Supplementary Information A, Fig. SA3). Bootstrap analyses were performed to assess variation in our estimates of the contribution of ITV and SPT (Fig. 2, solid line). The variance in community weighted means of body

size attributable to SPT and to ITV was assessed as the ratio of the regression sum of squares of the SPT model (SSR_{SPT}) or the ITV model (SSR_{ITV}) over the total sum of squares of the model including both SPT and ITV ($SST_{SPT+ITV}$). This quantifies the contribution of SPT and ITV, respectively, to the total explained variance in community average body size along the urbanization gradient. The proportional variation explained by ITV was determined using the formula: $SSR_{ITV}/(SSR_{ITV} + SSR_{SPT})$ (Lajoie & Vellend, 2015).

Genetic variation and phenotypic plasticity in body size in Daphnia magna

We tested for the effect of urbanization and temperature exposure on genotypic values (this term is clarified in Supplementary Information A, Box SA1) of *D. magna* size at maturity with clone (nested in population) and population included as random effects in our linear mixed-effect model (using the 'lme4' and 'car' packages in R to compute approximate *F*-test statistics and *p*-values for fixed effects; Fox & Weisberg, 2010; Bates *et al.*, 2014; R development core team, 2015). Built-up area was log-transformed. We applied the restricted maximum-likelihood estimation method (REML), and corrected the degrees of freedom for fixed effects using the Kenward–Roger approximation. Tests of normality were performed and assumptions were met (Supplementary Information A, Material and Methods). Significance of random effects was tested by model comparison (Wald's χ^2 and *p*-values computed using the 'car' package).

The relative contribution of genotypic trait variation, non-genetic intraspecific trait variation and interspecific trait turnover to the change in cladoceran community body size

To disentangle the relative contribution of genotypic trait variation (GTV) and non-genetic trait variation (i.e. phenotypic plasticity or ontogenetic shifts; $ITV_{PLASTICITY/OTHER}$) to the change in average community body size along the urbanization gradients, we modified the method of Lajoie & Vellend (2015) and included the genotypic trait values for 12 *D. magna* populations at 20 °C and 24 °C. Of the 10 communities containing *D. magna* in the community dataset, seven communities were shared between both datasets. For the other three *D. magna* communities, genotypic trait values were estimated using the regression function from the common garden experiment (Supplementary Information A). We determined the contributions of genotypic (GTV) and non-genetic trait variation along the urbanization gradient, by rewriting the phenotypic trait value z_i for a species *i* as the sum of its genotypic trait value z_i^G, its plasticity response to temperature ($ITV_{PLAST\text{-}T}$; $z_i^T - z_i^G$) and its plasticity response to other environmental conditions present in the field or ontogenetic changes (ITV_{OTHER}; $z_i - z_i^T$), i.e.:

$$z_i = z_i^G + (z_i^T - z_i^G) + (z_i - z_i^T) \quad (1.1)$$

with z_i^G the genotypic trait value calculated as the average of the trait values in the 20 °C and 24 °C treatments, and z_i^T the estimated effect of plasticity in response to temperature (*T*), based on an

expected relationship between percentage of built-up area and temperature (detailed in Supplementary Information, A). Substituting equation (1.1) into the ITV model of Lajoie & Vellend (2015) results for each in community *j* consisting of s_j species in

$$\sum_{i=1}^{s_j} q_{ij}(z_{ij} - \bar{z}_i) = \sum_{i=1}^{s_j} q_{ij}(z_{ij}^G - \bar{z}_i^G) + \sum_{i=1}^{s_j} q_{ij}\Big([z_{ij}^T - z_{ij}^G] - [\bar{z}_i^T - \bar{z}_i^G]\Big) + \sum_{i=1}^{s_j} q_{ij}\Big([z_{ij} - z_{ij}^T] - [\bar{z}_i - \bar{z}_i^T]\Big). \quad (1.2)$$

To calculate the abundance-weighted average community body size for these 10 communities, we used the genotypic trait values of *D. magna,* combined with the metacommunity-wide body sizes for all other species present in the local communities. As a result, the effects of ITV and GTV only reflect variation in the focal species *D. magna*. To determine the contribution of GTV, temperature-related phenotypic plasticity and phenotypic variation due to other environmental conditions or demographic population structure along the urbanization gradient, a regression analysis was performed on each of the three terms in the right-hand side of equation (1.2). To determine the proportional contribution of each trait component (GTV, $ITV_{PLAST\text{-}T}$, ITV_{OTHER} or SPT) a similar formula was used as in Lajoie & Vellend (2015). Two communities identified as outliers were excluded from this analysis; the results including these communities are presented in Supplementary Information B.

RESULTS

Intraspecific trait variation versus interspecific trait turnover

For the set of 83 communities (one outlier community removed, Fig. SA3 shows plots with outliers included), we found a significant negative relation between the abundance-weighted average community body size and percentage built-up area, but only when we quantified built-up area at small spatial scales (50 m, $\beta_1 = -0.077$, $p < 0.001$, Fig. 1a; 100 m, $\beta_1 = -0.059$, $p = 0.006$; other spatial scales $p > 0.05$; Supplementary Information A, Table SA5 and Fig. SA4). We observed a decrease in the contribution of variation explained by SPT to the total variation in community body size along the urbanization gradient with increasing spatial scale at which urbanization was determined (Fig. 2a, Supplementary Information A, Table SA5). We observed a slight increase in the contribution of variation explained by ITV at larger spatial scales, with highest contributions at the 200–800 m scales (Fig. 2b, Supplementary Information A, Table SA5). The relative importance of ITV versus SPT increased with spatial scale, but the impact of ITV was not significant (Fig. 2c, Supplementary Information A, Table SA5).

For the set of communities dominated by small species, a similar significant negative relation between abundance-weighted average community body size and percentage built-up area was found at smaller spatial scales (50 m, $\beta_1 = -0.068$, $p < 0.001$, Fig. 1c; 100 m, $\beta_1 = -0.057$, $p < 0.001$; 200 m, $\beta_1 = -0.051$, $p = 0.007$; other spatial scales $p > 0.05$; Supplementary Information, Table SA5 and Fig. SA4). SPT accounted for 23.34% to 11.76% of the total variation in average community body size along the urbanization gradient at small spatial scales, but declined rapidly to 0.21% at the largest spatial scale (Fig. 2d; Supplementary Information, Table SA5). There is a tendency for ITV to explain a larger portion of the observed average community body size turnover along the urbanization gradient at smaller spatial scales (but marginally non-significant, even at the 50 m scale; 1.73%, $p = 0.052$; Fig. 2e; Supplementary Information A, Table SA5). The relative importance of ITV to the combined contribution to variation explained by ITV and SPT accounted for 6.91% at this scale and increased with increasing spatial scale.

For the set of communities dominated by large species, we observed a very different pattern, with an increase in average community body size with percentage built-up area, which was only significant when built-up area is assessed at the larger spatial scales (400 m, $\beta_1 = 0.057$, $p = 0.046$; 800 m, $\beta_1 = 0.066$, $p = 0.034$; 1600 m, $\beta_1 = 0.085$, $p = 0.020$; 3200 m, $\beta_1 = 0.110$, $p = 0.024$, Fig. 1f; scales below 400 m $p > 0.05$; Supplementary Information A, Table SA5 and Fig. SA4). For this subset of communities, ITV explained a significant portion (8.48–11.63%) of the turnover in observed average community body size along the urbanization gradient at larger spatial scales (400–1600 m; Fig. 2h; Supplementary Information A, Table SA5). At all spatial scales except 3200 m, ITV explained more variation along the urbanization gradient than SPT.

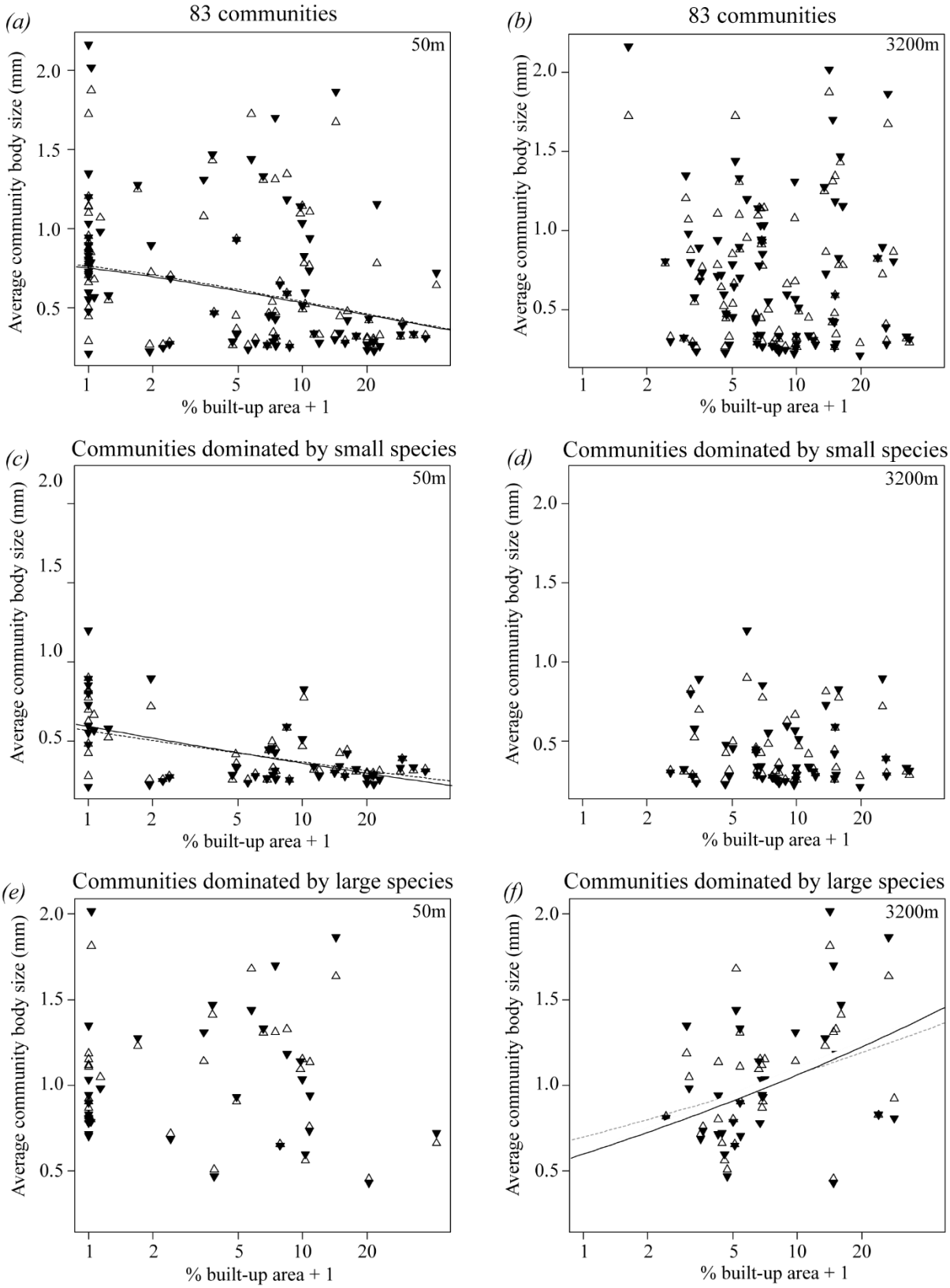

Figure 1. Relationships between body size variation and percentage built-up area (%BA + 1, plotted on a log-scale) among the 83 communities (a,b), the subset of communities dominated by small species (n = 49; c,d) and the subset of communities dominated by large species (n = 32; e,f), where average community body size is calculated using the local trait values of the species (filled triangles) and using the metacommunity-wide species trait values (unfilled triangles). Results are given for urbanization assessed at the local (50 m; a,c,e) and regional (3200 m; b,d,f) spatial scale. Significant relationships ($p < 0.05$) between body size variation and percentage built-

up area for the community body sizes calculated using the local and metacommunity-wide species trait values are shown by black solid and dashed lines, respectively. $p < 0.1$ are shown in grey lines. p-values are given in Supplementary Information A, Table SA5.

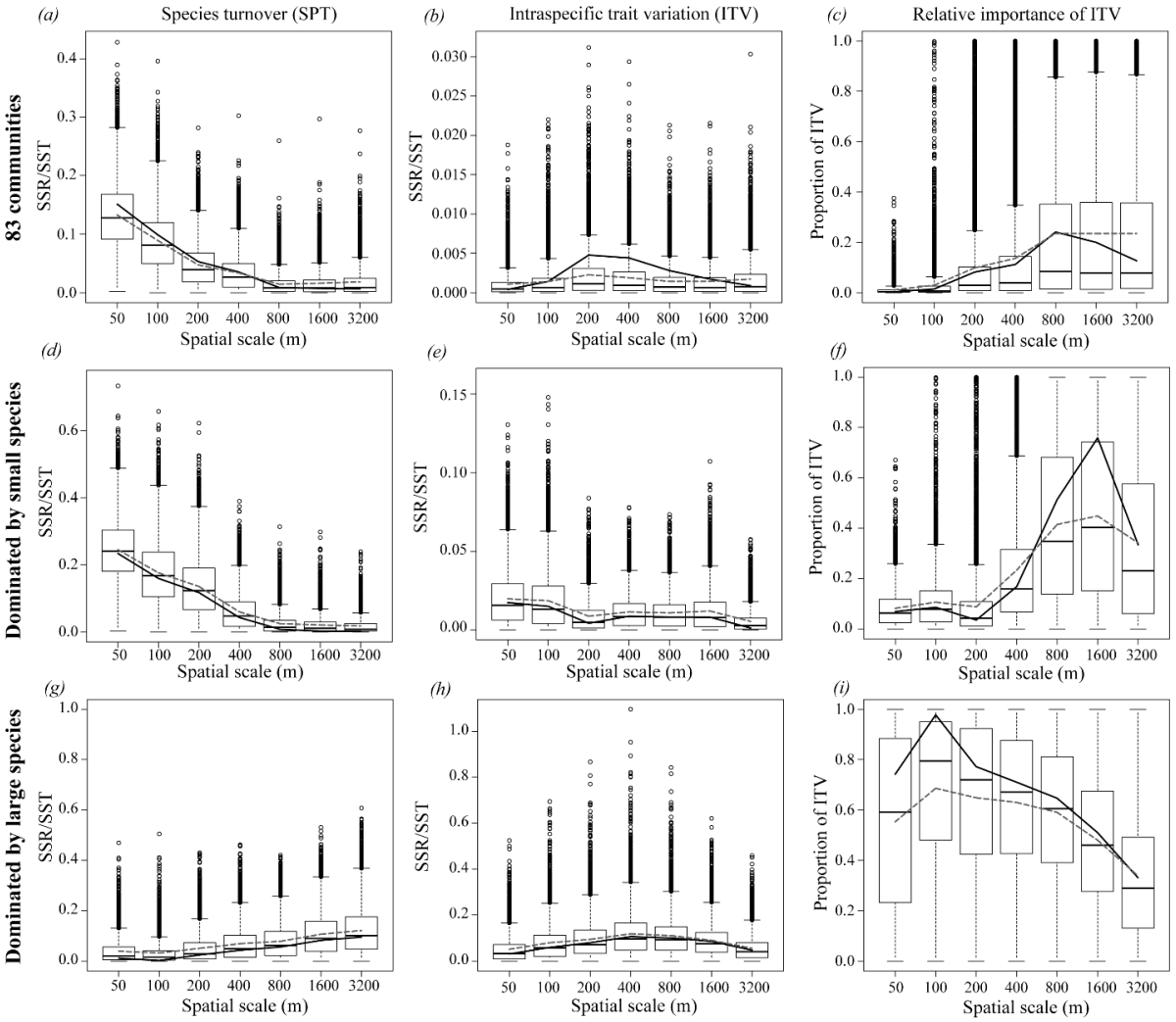

Figure 2. Bootstrap results of partitioning intraspecific and interspecific sources of body size variation along the urbanization gradient as quantified at different spatial scales for the total set of 83 communities (a–c), the subset of communities dominated by small species (n = 49; d–f) and the subset of communities dominated by large species (n = 32; g–i). The black solid line represents the variability of the total model explained by species turnover (SPT; a,d,g) or intraspecific trait variation (ITV; b,e,h) of the observed data sample and is calculated by dividing the regression sum of squares of the SPT model (SSR_{SPT}) or the ITV model (SSR_{ITV}) by the total sum of squares (SST) of the SPT + ITV model. The relative explanatory power of ITV to community body size turnover along the urbanization gradient is calculated as $SSR_{ITV}/(SSR_{ITV} + SSR_{SPT})$ and is for the observed data sample given by the black solid line (c,f,i). The dashed lines represent the average of 10.000 bootstrap samples. Box plots show the distributions of the bootstrap estimates.

Evolution and phenotypic plasticity of body size in *Daphnia magna*

Genotypic trait values for size at maturity of *D. magna* populations decreased with increasing levels of urbanization ($\beta_1 = -0.123 \pm 0.027$ SE, $F_{1/8} = 14.730$, $p = 0.004$; Fig. 3). A significant temperature effect was found, with individuals cultured at 24 °C having an average smaller size at maturity ($F_{1/273} = 6.432$, $p = 0.012$) than individuals cultured at 20 °C. In addition, there was a significant interaction effect between temperature and built-up area ($\beta_1 = 0.054 \pm 0.019$ SE, $F_{1/273} = 8.221$, $p = 0.004$; Fig. 3). Some of the more urban populations showed a similar to even larger size at maturity at 24 °C compared with 20 °C, while rural populations showed a clear reduction in size at maturity at 24 °C (Fig. 3). Moreover, the difference in size at maturity between 20 °C and 24 °C was larger in rural than in urban populations. Model comparison showed a significant effect of clone ($\chi^2_1 = 14.444$, $p < 0.001$) and population ($\chi^2_1 = 4.376$, $p = 0.036$), which were included as random factors in the model. Clonal temperature reaction norms for each population along the urbanization gradient are shown in Supplementary Information A, Fig. SA5).

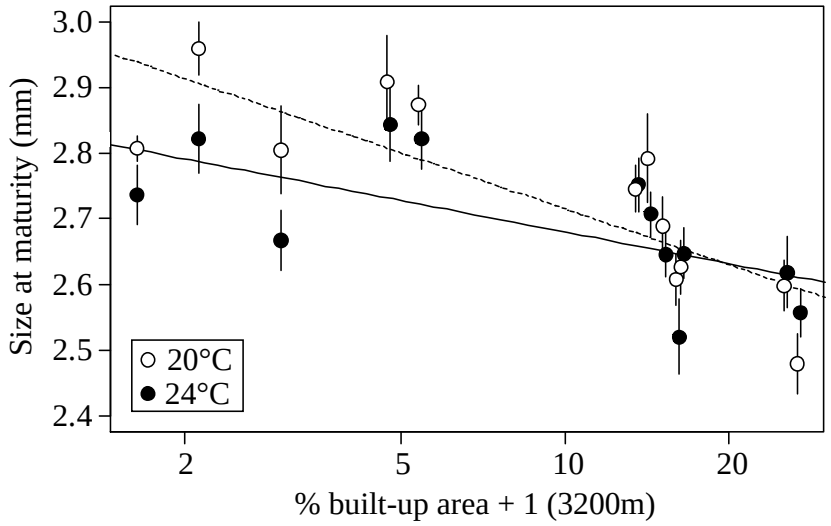

Figure 3. Regression of average population genotypic values of size at maturity (mm) ±1 SE. of 12 D. magna populations along percentage built-up area (%BA + 1, assessed at the 3200 m scale, plotted on a log-scale). Empty symbols and the dotted line denote size at maturity at 20 °C, while filled symbols and the solid line refer to size at maturity at 24 °C as determined in a common garden experiment using two to six clones per population.

The relative contribution of genotypic trait variation, non-genetic intraspecific trait variation and interspecific trait turnover

For communities in which *D. magna* was present, a tendency towards an increased average community body size was detected with increasing levels of urbanization (Fig. 4a, 3200 m scale), similar to the pattern found for the total set of communities dominated by large species. Owing to the low number of communities, however, the full model was not significant (SPT + ITV, p = 0.423, Supplementary

Information A, Table SA6; for results without outlier removal, see the Supplementary Information B, Table SB5 and Fig. SB8). ITV of *D. magna* explained 32.43% of the total observed trait turnover along the urbanization gradient (marginally non-significant, p = 0.056, Supplementary Information A, Table SA6). ITV was clearly the larger contributor, explaining 95.78% of the total variation explained by both ITV and SPT together. Including genotypic trait values at 20 °C and 24 °C in the analysis enabled us to disentangle three types of ITV along the urbanization gradient: genotypic trait variation (GTV), non-genetic phenotypic trait variation due to increased temperatures ($ITV_{PLAST\text{-}T}$) and non-genetic phenotypic trait variation due to other environmental gradients or ontogenetic changes (ITV_{OTHER}; Fig. 4b). Only the latter component showed a significant contribution to the observed increase in average community body size (Supplementary Information A, Table SA6, p = 0.047). ITV_{OTHER} explained 92.89% of the total explained variation by all trait components, while GTV and SPT contributed 4.55% and 2.56%, respectively. Regression analysis on the difference in *D. magna* body size measurements on all individuals (i.e. including juveniles) versus adults only plotted against degree of urbanization, suggests that the large impact of ITV_{OTHER} might be due to a change in relative abundance of juveniles along the urbanization gradient (β_1 = 20.048, p = 0.032; Supplementary Information A, Fig. SA6).

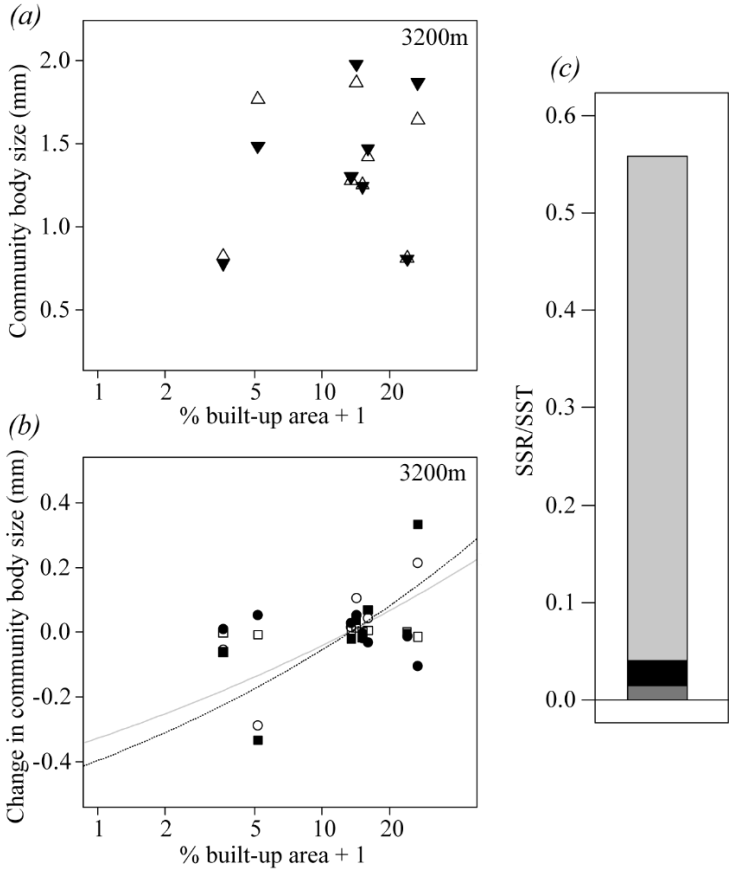

Figure 4. (a) Relationship between average community body size variation and percentage built-up area (%BA + 1, 3200 m, plotted on a log-scale) of eight D. magna communities (two outliers were removed, PL25-red and TP-Blap1-riv similar to the general analysis of all large-species dominated communities). Community average body size is calculated using the local trait values of *D. magna* (filled triangles; note: for all other species we used community-wide average body size values) or the community-wide average *D. magna* body size (unfilled

triangles). (b) Relationships between intraspecific trait variation (ITV, unfilled circles, grey solid line, $p < 0.1$), genetic trait variation (GTV, filled circles), plasticity response to temperature ($ITV_{PLAST-T}$, unfilled squares), and plasticity response to other cues (ITV_{OTHER}, filled squares, black dotted line, $p < 0.05$) with percentage built-up area (%BA + 1, 3200 m, plotted on a log-scale). p-Values are given in Supplementary Information A, Table SA6. (c) Visualization of the relative importance of SPT (dark grey), GTV (black), $ITV_{PLAST-T}$(not visible due to its small value) and ITV_{OTHER} (light grey) to the total amount of variation in community body size along the urbanization gradient explained by all components of ITV and SPT.

DISCUSSION

We observed an overall decrease in average community body size of cladoceran communities in response to urbanization. The overall pattern as well as the relative contribution of intra- and interspecific trait variation to this pattern depended, however, strongly on the spatial scale at which urbanization was quantified and whether or not the communities were dominated by large species. For one of these large species, *D. magna*, a common garden experiment revealed a significant genetic decline in size at maturity with increasing levels of urbanization around the ponds. In addition, the animals matured at a smaller size when reared at 24 °C compared with 20 °C. Disentangling the effects of all intraspecific trait components (i.e. GTV, plasticity in response to temperature, plasticity related to other environmental variables and ontogenetic shifts) revealed that the community trait change along the urbanization gradient was mainly driven by intraspecific trait shifts not related to genotype or temperature.

In our dataset of 83 ponds, the average cladoceran community body size declined with increasing urbanization, which is in line with our expectations given that urban areas represent heat islands (Oke, 1973; De Ridder *et al.*, 2015) and that higher temperatures favor smaller body sizes (Angilletta *et al.*, 2004; Kozłowski *et al.*, 2004). Our pilot temperature logging study of ponds along the urbanization gradient offers the first additional evidence of aquatic ecoystems being impacted by the urban heat island effect as the average summer (July–August) maximum water temperature of 2014 differed by 4.03 °C between two of the most urban and two of the most rural ponds along the gradient (Supplementary Information A, Fig. SA2). The decrease in community body size was only significant for built-up area assessed in the immediate surroundings of the pond (50–200 m radius). This most likely reflects built-up area in the immediate vicinity of the pond being the best predictor of extreme temperatures, while parks and large gardens in the cities experience somewhat cooler temperatures (Yu& Hien, 2006; Ren *et al.*, 2013; Alavipanah *et al.*, 2015). We, however, found that cladoceran communities characterized by a relatively high relative abundance (more than 5%) of large-bodied species displayed a different pattern. Average community body size of these communities increased with increasing built-up area at larger spatial scales (400 m and more). This pattern is opposite to the pattern found for the total set of communities and for the communities dominated by small-bodied species. We can only speculate on why we find an opposite pattern for communities dominated by large-bodied species. One possible explanation might be that urban ponds on the one hand tend to be rich in

nutrients (Kaye *et al.*, 2006) while, on the other hand, by being relatively isolated they can be characterized by a reduced presence of zooplanktivorous fish or increased relative importance of small, gape-limited invertebrate predators. These factors would favour large-bodied zooplankton (Dodson, 1974; Rabus *et al.*, 2011). The fact that this pattern is only observed when urbanization is quantified at larger spatial scales (400 m radius or more) reflects regional effects, and is in line with the predator hypothesis. Dispersal barriers can prevent specific predators being present; this would result in urbanization effects acting at a larger scale independently of the presence of built-up area in the immediate vicinity of the study habitats. Ponds in city parks or other urban green infrastructure are indeed still relatively isolated from each other, compared with rural ponds.

We used the variation partitioning method described by Lajoie & Vellend (2015) to assess the relative importance of intra- versus interspecific trait variation to the observed community body size turnover along the urbanization gradient. For the total set of communities as well as for the communities dominated by small-bodied species, we found significant contributions of variation explained by SPT and ITV when urbanization was assessed at small spatial scales, with SPT being the more important contributor to explained variation. For the communities dominated by small-bodied species, the relative contribution of ITV to the total body size turnover along the urbanization gradient increased at larger spatial scales (400 m and more), but at these scales the regression models were non-significant. For communities dominated by large-bodied species, the contributions of SPT and ITV to body size turnover along the urbanization gradient were of the same order of magnitude at larger spatial scales (ITV accounted for 51% of the total amount of explained variation at 1600 m and 33% at 3200 m). The contribution of ITV, however, became even larger at intermediate (71% at 400 m and 65% at 800 m; ITV models significant) and smaller spatial scales (even up to 98% at 100 m).

For the large-bodied water flea *D. magna*, we quantified genetic variation in size at maturity in a common garden experiment. As predicted, we found that body size declined with increasing levels of urbanization. This result is in line with other studies that provided evidence for reductions in body size with increasing urbanization in carabid species (Weller & Ganzhorn, 2004) and a few other terrestrial organisms, such as house sparrows (*Passer domesticus*; Liker *et al.*, 2008) and tropical frogs (*Dendropsophus ebraccatus;* Matías-Ferrer & Escalante, 2015). The opposite pattern of increased body size with higher levels of urbanization has, however, been reported for the orb weaving spider *Nephila plumipes* (Lowe *et al.*, 2014). Whereas these former studies focused on patterns of phenotypic trait variation, our study is the first to show a genetic based reduction in body size along rural–urban gradients in aquatic ecosystems. We also observed a significant phenotypic plasticity response to temperature, with *D. magna* individuals maturing at a smaller size when reared at 24 °C compared with 20 °C. This observation is in line with expectations based on the urban heat island effect and oxygen constraints (Atkinson, 1994). Especially in aquatic organisms, a reduction in body size with increasing temperatures can be necessary, as oxygen is limited in supply in aquatic systems and its solubility in

water decreases while organismal metabolic rates increase with increasing temperature (Horne *et al.*, 2015). The temperature loggers in the two urban and two rural ponds yielded an average temperature difference during the summer months (July–August) of approximately 4.03 °C (see Supplementary Information A, Fig. SA2). Assuming an urban heat island effect of approximately 4 °C, the combined effect of genetic based change and phenotypic plasticity would result in a total reduction in size at maturity of almost 10% (from 2.87 mm to 2.60 mm) in urban compared with rural populations. Finally, we also observed a genotype by environment interaction between population (different populations along the urbanization gradient) and phenotypic plasticity in response to rearing temperature. This likely reflects a pattern of local adaptation through the evolution of phenotypic plasticity. More specifically, while the urban populations show a clear pattern of reduced mean size at maturity compared with rural populations, their phenotypic plasticity in size at maturity in response to increased temperature is much less strong. Our study adds to the evidence of pronounced genetic differentiation and local adaptation of natural *Daphnia* populations over short time periods and at microgeographic scales in relation to natural and human-induced environmental changes (Geerts *et al.*, 2015; Stoks *et al.*, 2016). We also provide evidence of specific urbanization-driven evolutionary change, as has been reported for a few other species (Partecke & Gwinner, 2007; Tüzün *et al.*, 2015).

Modifying the variation partitioning method used in Lajoie & Vellend (2015) enabled us to integrate all three levels of trait variation (individual plasticity, genotype-dependent trait values and species-specific trait values) that shape eco-evolutionary dynamics along environmental gradients (Violle *et al.*, 2012). Assessing the contribution of GTV, phenotypic plasticity in response to a given environmental condition (here temperature; $ITV_{PLAST\text{-}T}$) and a rest component of ITV (reflecting phenotypic plasticity to unmeasured environmental conditions or ontogenetic variation linked to individual growth and development; ITV_{OTHER}) to the observed community body size turnover, led to three important conclusions. First, most variation in average community body size along the studied urbanization gradient was due to ITV rather than SPT. Second, among the components of ITV, the largest contributor was the component linked to phenotypic plasticity induced by other environmental variables than temperature and those reflecting ontogenetic growth. The latter explanation is more likely as our measurements of body size in the field samples involved randomly sampled individuals and thus were not limited to adults. There is a large difference in body size between neonate (approx. 0.6–0.7 mm) and adult (up to 5 mm) *D. magna* (Ebert, 1991), so that differences in longevity, age distribution and the relative abundance of adults and juveniles can have an important impact on average community body size. Third, we found an opposite pattern of increasing body size with urbanization in field collected *D. magna* compared with the decreasing genotypic values of body size with increasing levels of urbanization in the same species. This provides evidence for phenotypic and genotypic countergradient variation for body size (Conover & Schultz, 1995) along an urbanization gradient in this species, and would lead to an idiosyncratic pattern of the observed ITV along the urbanization

gradient (Albert *et al.*, 2010). We note that the phenotypic trait shift observed in the field likely reflects an ontogenetic shift and is not due to phenotypic plasticity in response to temperature, as this latter response positively covaried with the genetic differences. We speculate that the countergradient genetic shift to smaller size at maturity in urbanized areas is important for survival of the *D. magna* populations in urban ponds. This genetic response may allow survival at higher temperatures in an environment that simultaneously leads to larger individuals by enabling longer lifespans (e.g. due to reduced predation) in this species that is characterized by indeterminate growth. While genetic changes only contributed less than 5% to the total change in community body size along the urbanization gradient, evolution still accounted for double the percentage of variation explained by SPT. Our analysis also reveals a case of hidden evolution, i.e. evolution obscured by ecological change as measured in the field. This reflects the complex interplay between environmental, ecological, phenotypic and genotypic processes, often leading to cryptic eco-evo dynamics (Kinnison *et al.*, 2015).

Zooplankton are key grazers of phytoplankton in ponds and lakes (Jeppesen *et al.*, 1999). Small-bodied zooplankton are less efficient grazers than large-bodied species, especially large-bodied *Daphnia* species (Brooks & Dodson, 1965; Dodson, 1974; Tessier *et al.*, 2000). Recent experimental work with Belgian zooplankton species confirmed that assemblies of large cladoceran species can have an up to sevenfold higher impact on phytoplankton biomass than assemblies of small cladoceran species of the same biomass (Gianuca *et al.*, 2016). The here observed reduction in average body size with increasing urbanization at the level of the community, as well as in terms of genotypic values at the population level and phenotypic plasticity at the level of individual genotypes, may therefore lead to reduced top-down control of phytoplankton, increasing the probability of a shift to the turbid, phytoplankton dominated state (Scheffer *et al.*, 1993; 2001). Moreover, in combination with the urban heat island effect and an increased number of extreme temperature events, the reduced capacity to control blooms of toxic cyanobacteria such as *Microcystis* (Kosten *et al.*, 2012) could generate health risks.

Knowledge gaps and future directions

Overall, our results show that ITV can be an important driver of total community body size turnover along strong environmental gradients, in this case urbanization. Depending on the spatial scale at which urbanization was quantified, 50% or more of community turnover in cladoceran body size could be explained by ITV. We also showed that the large-bodied cladoceran *D. magna* genetically adapts to the degree of urbanization and remains smaller at maturity in ponds in urbanized areas. In real landscapes, this effect on body size is expected to be even more pronounced given that the animals also show phenotypic plasticity for smaller size at maturity with increasing temperatures. Yet, our analysis of the size distribution of *D. magna* individuals in samples taken from the field shows an increase in body size with increasing urbanization, which might reflect changes in age distribution along the urbanization

gradient. In our integrated analysis, it was this phenotypic increase in body size of *D. magna* with increasing urbanization that was the major determinant of the urbanization-driven change in average community body size. Genotypic trait change contributed relatively little to the overall change in average community body size along the rural–urban gradient. Yet the observed genetic trait shift towards smaller sizes at maturity with increasing urbanization is likely important to the success of *D. magna* in urban ponds, as the countergradient evolution enables this species to deal with the higher temperatures in settings that support a phenotypic shift to larger individuals. Our integrated analysis revealed striking patterns of body size turnover along urbanization gradients in response to the urban heat island effect, mediated by evolutionary and non-evolutionary ITV as well as by changes in the relative abundances of species. Moreover, we detected a number of surprises and complexities such as the differences in responses between communities dominated by small versus large species, the cryptic eco-evolutionary dynamics, and the fact that ITV likely associated with ontogenetic shifts in one species has a stronger impact on community trait turnover than shifts in species composition. These complexities need to be considered in future studies and can strongly impact predictions of trait shifts in the face of environmental change.

Our extended partitioning approach allows combining field data with detailed quantitative genetic analyses obtained from common garden experiments. It can be applied to any trait and provides a highly valuable tool to study eco-evolutionary dynamics along environmental gradients in time and space. In this study, we integrated genotypic and phenotypic data of one species, but ideally, GTV of all important member species of the community should be included to better grasp the interplay between evolutionary and non-evolutionary drivers of ITV and their relative importance to SPT in explaining community responses to environmental gradients.

Our analyses are limited to one sampling campaign. We acknowledge that including multiple data points through time would likely provide more power to detect the underlying drivers of the observed trait change along the urbanization gradient and correct for seasonal variation. This could help in generating more specific predictions on the causes and consequences of community trait shifts associated with urbanization. Our results indicate that the eco-evolutionary dynamics may have important consequences for ecosystem functions and ecosystem services provided by urban ponds. Quantifying these ecological and socio-economic consequences of ITV in urban ponds was beyond the scope of the present study, but should be tackled in future work.

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Chapter 6:

Cryptic eco-evolutionary dynamics in the city: evolution of prey buffers the effect of evolution of the predator in an aquatic food chain

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Abstract

It is becoming increasingly clear that urbanization drives rapid evolution in plant, animal, and microbial species. Yet, how urban evolution might impact ecological dynamics is so far poorly understood. Damselflies and water fleas show adaptive evolution directly and indirectly linked to thermal adaptation in response to anthropogenic warming associated with urban city centers. We conducted a large-scale common garden experiment to test whether the evolutionary background of damselflies and water fleas (urban vs. rural) shapes the outcome of predator-prey interactions under thermal stress (simulated heatwaves). Urban and rural damselfly predators (*Ischnura elegans*) and water flea prey (*Daphnia magna*) were reared at 20 and 24 °C and exposed to simulated mild and intense five-day heatwaves (+ 4 °C and +8 °C temperature increases), after which predation rates were assessed. Urban *Daphnia* were overall significantly smaller compared to rural *Daphnia*, and when reared at higher temperatures (20 vs. 24 °C). In general, heatwave intensity impacted predation efficiency of damselflies negatively. The impact of an increased rearing temperature was higher than the intensity of the heatwave. When focusing on urban/rural contrasts, correcting for possible covariates such as water flea and damselfly body mass, predation rates were significantly impacted by the urbanization background of both interactors. Urban damselflies showed higher predation rates compared to rural damselflies, but only when encountering rural *Daphnia*. When predating on urban *Daphnia* genotypes, no difference was observed between urban and rural damselfly predation rates, indicating higher survival of urban *Daphnia* when exposed to urban damselflies. This increase in predation linked to a 'mismatch' between the urbanization level of predator and prey (urban predator – rural prey) was not present for rural predators. These results provide evidence that evolution driven by urbanization feeds back to ecology, in this case predator-prey dynamics, which might substantially alter food web dynamics. For example, large water fleas such as *Daphnia magna* are important for stabilizing clear water states in small freshwater ecosystems. Higher survival when encountering urban damselflies might guarantee population survival and thus positively feed back to algae top down control in urban ponds.

INTRODUCTION

Human impacts on micro-evolutionary processes have been widely documented over the past decades (Wu & Kruckeberg, 1985; Palumbi, 2001; Stockwell *et al.*, 2003; Berteaux *et al.*, 2004; Pulido & Berthold, 2004; Davis *et al.*, 2005; Geerts *et al.*, 2015; Hendry *et al.*, 2017; Kuparinen & Festa-Bianchet, 2017). An important human-associated driver of evolution is urbanization (Donihue & Lambert, 2015; Johnson & Munshi-South, 2017). A recent burst of studies show urbanization drives evolutionary changes in a wide range of plant (Cheptou *et al.*, 2008; Yakub & Tiffin, 2016; Thompson *et al.*, 2016), animal (Harris *et al.*, 2013, 2016; Winchell *et al.*, 2016), and micro-organism (McLean *et al.*, 2005) populations, including both neutral as well as adaptive evolution (Johnson & Munshi-South 2017), and spanning both indirect (e.g. domestication of animals: Arnold, 2004; Driscoll *et al.*, 2009; industrialization-driven melanization of moths: Cook & Saccheri, 2013,) and more direct urbanization-associated processes (e.g. thermal adaptation: Brans *et al.*, 2017b; pesticide-adaptation: Tüzün *et al.*, 2015; locomotory adaptation to man-made surfaces: Winchell *et al.*, 2016, 2018; adaptation to habitat fragmentation: Cheptou *et al.*, 2008).

Given the fast rate at which evolution can take place (Thompson, 1998; Hendry & Kinnison, 1999; Hairston *et al.*, 2005), the existence of 'eco-evolutionary dynamics' have now been widely recognized in evolutionary biology and ecology (Hairston *et al.*, 2005; Schoener, 2011; Urban *et al.*, 2008; Pelletier *et al.*, 2009; Hendry, 2017). One part of the 'eco-evolutionary equation' suggests (adaptive) evolution impacts inter- and intraspecific ecological dynamics, such as competition, predation, mutualistic and host-parasite interactions. This feedback potentially underlies shifts in community composition along environmental disturbance gradients that can affect ecosystem functions (Yoshida *et al.*, 2003; Fussmann *et al.*, 2007; Johnson *et al.*, 2009; Alberti *et al.*, 2017b; Hendry, 2017). While theoretical studies, and review and concept papers, on eco-evolutionary feedback loops are numerous (Fussmann *et al.*, 2007; Moya-Laraño *et al.* 2012; Vanoverbeke *et al.*, 2016; Bocedi *et al.*, 2014; Patel & Schreiber, 2015; Hendry, 2017; Patel *et al.*, 2018), empirical evidence on evolution feeding back to ecological dynamics in nature is more limited due to the intrinsic complexity associated with studying such dynamics in nature (Bassar *et al.*, 2009; Johnson & Agrawal, 2007; Johnson *et al.*, 2009; Farkas *et al.*, 2013, and the work on community genetics; Whitham *et al.*, 2006, 2010). In addition, selection pressures are expected to impact not just one, but multiple interacting species across trophic levels in a local community. Empirical studies on eco-evo feedbacks integrating the evolutionary trajectory of both predator and prey are (we note that we here exclude specific co-evolutionary arms race studies; Thompson, 2005; e.g. Decaestecker *et al.*, 2007, 2013; and the study on microbial species by Celiker & Gore, 2014). Obtaining insight in eco-evolutionary feedbacks multi-species framework is a necessary step forward in eco-evolutionary dynamics research to improve the forecast on how communities will responds to challenges of the Anthropocene (Urban *et al.*, 2016).

While eco-evolutionary feedbacks have received substantial attention in the framework of climate change (Norberg *et al.*, 2012; Urban et al, 2012; Bailey, 2013; Urban *et al.*, 2016; Hendry, 2017a), studying responses to urbanization might be of substantial value to disentangle evo-to-eco feedbacks (Alberti, 2015; Alberti *et al.*, 2017b). Urbanization is associated with fast environmental change (Kaye *et al.*, 2006; Parris, 2016), replicated on a global scale, inducing rapid evolutionary change in many species (Donihue & Lambert, 2015; Johnson & Munshi-South, 2017). Alberti (2017b) made a strong case reviewing urban genotypic and phenotypic trait changes, how they are linked to ecological processes, and thus possibly impact ecosystem functions. Nevertheless, empirical evidence or attempts to assess specific feedbacks in urban settings are still limited (Brans et al, 2017a; Hendry *et al.*, 2017b).

Recent work on the water flea *Daphnia magna* revealed that urbanization in Flanders (Belgium) drives genetic differentiation in a suit of traits including heat tolerance (Brans *et al.*, 2017b), life history (Brans *et al.*, 2017a; Brans & De Meester, 2018), and physiology (Brans *et al.*, 2018b). When reared under common garden settings, urban water flea genotypes show an upregulation of heat tolerance with ca. 2 °C (Brans *et al.*, 2017b) and a faster life style driven by fast development, a smaller size at maturity (Brans *et al.*, 2017a; Brans & De Meester, 2018), and a higher fecundity. Urban *Daphnia* also have evolved higher constitute levels of energy molecules (fat, protein, sugar content) and a more efficient regulation of antioxidative stress responses (Brans *et al.*, 2018b). Strikingly, physiological parameters such as energy reserves and oxidative stress responses were closely linked with life-history differentiation via a pace-of-life Syndrome (POLS; Rickleffs & Wickelski, 2002; Réale *et al.*, 2010; Sepp *et al.*, 2017) in urban, yet not in rural *Daphnia* populations (Brans *et al.*, 2018b). The latter finding aligns with the hypothesis that POLS are likely to occur in and evolve upon disturbance or stressful environmental changes (Dingemanse *et al.*, 2004; Killen *et al.*, 2013; Charmantier *et al.*, 2017; Segev *et al.*, 2017; Sprau *et al.*, 2017). Urban ponds in the study region (Flanders, Belgium) are warmer compared to rural ponds (up to 3.69 °C warmer in maximum daily temperature measured over 24h periods during summer, Brans *et al.*, 2018a), with temperature differences most pronounced in spring and summer. In addition, urban ponds often suffer from higher disturbance (mechanical disturbances such as vegetation clearance and water removal; Hassall, 2014; Blicharska *et al.*, 2016; Hill *et al.*, 2017; Thornhill *et al.*, 2018a,b; Suski *et al.*, 2018) and are often exposed to polluted run-off containing organic and chemical toxicants (e.g road salts, nutrients, pesticides, and heavy metals) and plastic debris (Paul & Meyer, 2001; Hassall, 2014; Hill *et al.*, 2017; Thornhill *et al.*, 2018a).

Parallel to the work on water fleas and in the same region (Flanders, Belgium), Tüzün *et al.* (2015, 2017a,b,c) reported evolutionary responses in activity patterns, development, and growth in damselflies. When reared along a thermal gradient in the lab, urban damselflies show higher survival, yet slower growth rates (cf. countergradient shift in Thermal Performance Curves; TPCs; Conover & Schultz, 1995; Conover *et al.*, 2009). These responses seem to be indirectly linked to the thermal

regimes experienced by damselflies in urban areas: the growing seasons in urban ponds is up to 45 days longer in comparison with rural ponds (Brans *et al.*, 2018a), which substantially reduces time stress inflicted upon urban compared to rural damselflies to develop into the pre-emergence larval stage before winter starts. Tüzün at al. (2015) additionally reported urban damselflies to increase activity upon exposure to pesticides in the laboratory, suggesting adaptive mechanisms present that enable higher functionality upon pesticide exposure in these populations. Lastly, in urban damselfly populations, similarly as seen for *Daphnia*, a POLS structured the correlation between boldness and activity, whereas this was absent in rural populations, confirming urbanization can both shape and decouple trait covariation patterns as observed in other species such as birds (Charmantier *et al.*, 2017; Sprau *et al.*, 2017; Sepp *et al.*, 2017; Sol *et al.*, 2018).

These studies suggest that both water fleas and damselflies have genetically adapted to urbanization in the study region. For *Daphnia* there seems to be a a direct link between the observed genetic differentiation in traits (e.g. heat tolerance) the urban heat island effect, whereas for damselflies the thermal environment shapes life-history and behavioral differentiation in a more indirect way via phenological changes. Such differential evolutionary trait responses to temperature of multiple species is expected to change the outcome of their interaction, as shown for consumer-resource dynamics (Deblock *et al.*, 2013; Dell *et al.*, 2014; Gilbert *et al.*, 2014; Öhlund *et al.*, 2015; Cheng *et al.*, 2017). Predator consumption rates as given by functional response curves (i.e. density-dependent feeding rates) are strongly temperature-dependent (Sentis *et al.*, 2013b; Wasserman *et al.*, 2016). This implies that the outcome of interactions between predator and prey will depend on the degree of thermal adaptation of both interacting species. De Block *et al.*, (2013) reported predation rates of damselflies (*Ischnura elegans*) on water fleas (*Daphnia magna*), isolated from three latitudes (France, Belgium, Sweden) and reared at two testing temperatures (20 and 24 °C) to strongly depend, not only on the testing temperature, but also on the latitude of origin of both predator and prey. However, such predator-prey dynamics have rarely been studied along microgeographical thermal gradients, such as urbanization. For example, Philpott & Bichier (2017) showed predation pressure on aphids to decrease with increasing urbanization, most likely due to direct effects of temperature on aphid population growth. Yet, so far, no study currently reports on how urban evolution in interacting species feeds back to their predator-prey interactions.

We here set out to test the hypothesis that urban evolution shapes the outcome of predator-prey dynamics in urban freshwater ecosystems by means of a common garden rearing experiment involving both *Daphnia magna* (prey) and *Ischnura elegans* (predator) populations originating from urban and rural settings. Urbanization is associated with a higher incidence of extreme temperature events such as heatwaves (Wouters *et al.*, 2017). Extreme temperature events are fundamentally different from increases in average temperatures as organisms are challenged at the extremities of their thermal tolerance range (given by CT_{MIN}-T_{OPT}-CT_{MAX}; Sinclair *et al.*, 2012; Sentis *et al.*, 2013a, 2016; Colinet

et al., 2015; Stoks *et al.*, 2017; Tüzün & Stoks, 2018b). We assessed predation rate under different heatwave scenarios by means of feeding trials in which urban and rural predators were cross-combined with urban and rural prey after specific five-day heatwave treatments. We included a heatwave treatment as it might elucidate patterns of thermal adaptation more clearly in both interactors and trigger evo-eco feedbacks. For example, predation rates often increase with increasing average temperatures, yet decrease rapidly at extreme temperatures as a result of decreased searching activities and attack rates, in combination with longer handling times (Sentis *et al.*, 2013b; Stoks *et al.*, 2017). In case of thermal adaptation to extreme temperature events, we hypothesize these responses to be different in urban compared to rural damselflies. Likewise, urban *Daphnia* might be less vulnerable to extreme temperatures given their higher heat tolerance, possibly rendering them less vulnerable for predation.

In this study, we prioritize on testing how urban evolution in two species (not local adaptation to specific pond characteristics) can alter the outcome of their interaction, which implies sampling independent populations of both predator and prey, and integrating multiple urban and multiple rural populations. This study, involving two species, two evolutionary backgrounds (urban/rural), and a dynamic thermal environment inflicting stressful conditions for both interactors, aimed at elucidating how urban evolution in two interacting species impacts ecological dynamics such as predator-prey interactions upon thermal stress.

MATERIAL AND METHODS

To assess what the impact is of the evolutionary background (urban vs. rural) on predator-prey interactions under thermal stress, we set up an experiment involving both urban and rural damselflies (*Ischnura elegans*) and water fleas (*Daphnia magna*). Animals were reared at 20 and 24 ° C. These temperatures resemble the natural conditions for urban and rural populations, thus mimicking those specific urban and rural temperatures observed in the field (urban and rural ponds approximately differ 4 °C during summer, with rural ponds averaging around 19 °C, and rural ponds around 23 °C during summer; Brans *et al.*, 2018a) Including different rearing temperatures enabled us to rear rural and urban populations at the temperatures mimicking conditions experienced in the field. Next, the animals were exposed to a +8 °C temperature increase (over a period of 5 days) mimicking a heatwave (relative to their own environmentally relevant rearing temperature). We added an additional heatwave increase of +4 °C for those animals reared at 24 °C in order to assess the importance of acclimation temperature to performance under thermal stress (see further). At the end of the heatwave, predation rates were assessed.

Collecting and rearing of *Ischnura elegans* and *Daphnia magna*

Both damselfly and water flea populations originated from replicated urban and rural populations sampled from ponds in the region of Flanders (Belgium; same populations as in Tüzün *et al.*, 2015; Brans *et al.*, 2017a,b). Ponds were classified as urban/rural based on the percentage built-up area (BA) in the regional surroundings of the pond (1600 m radius). Percentage built-up area was derived from the Large-Scale Reference Database, available from the Flanders Geographical Agency. Populations of both interactors did not originate from the same ponds, as we set out to test how urbanization-driven evolution, rather than adaptation to local pond conditions, would shape predator-prey interactions between damselflies and water fleas upon their encounter (De Block *et al.*, 2013).

Damselfly predators (Ischnura elegans)

Ischnura populations originated from three rural and three urban populations, and were collected late July to late August 2015. For each pond, between 5 and 17 actively mating females (copula observations) were collected and brought to the laboratory. Females were provided with wet filter paper in plastic 100ml trays where they were let to oviposit. Eggs were incubated at 22 °C and 14:10 L:D photoperiod (Tüzun *et al.*, 2015). After hatching, the larvae were isolated into individual cups filled with 200 ml of aged tap water to prevent cannibalism and to use as individual experimental units (damselfly individuals) in the later experiments. Individual larvae were placed in temperature-controlled water baths at 20 °C or 24 °C (± 0.5 °C) and were daily fed *Artemia* nauplii ad libitum). Photoperiod was set at a 14:10 L:D regime. Larvae were grown until the F0 stage (i.e. the stage just before emerging to terrestrial adult damselflies). F0-larvae were assigned to a specific 5-day heatwave treatment and predation trial (according to specific combinations with *Daphnia* clones, see further). Only F0 larvae younger than 8 days were used to prevent using larvae close to emerging; before emerging they stop feeding which would bias the predation assessment.

Water flea prey (Daphnia magna)

Pre-experimental culturing conditions

A total set of 24 *D. magna* clones were reared in the laboratory, originating from four urban and four rural ponds (three clones per population). Sampling was carried out in June-August 2013 and June 2015 (see also Brans *et al.*, 2017a,b; Gianuca *et al.*, 2018). Clones were isolated from an in situ live sample and were separately inoculated in the laboratory in 200ml glass jars and kept at 20 °C for several generations (pre-experimental culturing conditions). Clonal identity was screened by means of 15 specific microsatellite markers following Orsini *et al.* (2012; see also Brans *et al.*, 2017a), to assess clonal identity.

Experimental culturing conditions

After two generations of standard culturing conditions (20 °C; 16:8 L:D photoperiod; 100 000 cells/ml of the unicellular green algae *Acutodesmus obliquus*), for each clone, triplicate cohorts of 12 individuals were reared in dechlorinated tap water at both 20±1 °C and 24±1 °C in 500ml glass jars, under a continuous 14:10 L:D photoperiod. Animals were fed the green algae *Acutodesmus obliquus* in a concentration of 1 mg $C.l^{-1}$ or 100.000 $cells.ml^{-1}$. Water was refreshed every two days. Cohorts were cultured for at least two generations at these conditions (i.e. 20 and 24 °C; 14:10 L:D), after which newborn juveniles (< 24h old) of second up to fifth clutch were used for the experimental heatwave treatment and the following predation trial. Thirty newborn juveniles were transferred into white plastic trial aquaria filled with 1 liter of fresh dechlorinated water and let to acclimatize for 30 minutes before entering the heatwave treatment. Newborn juveniles (< 24h old) were used for inoculation at the start of the heatwave (HW) treatments, as this avoids the risk of juveniles maturing and reproducing at an early stage into the heatwave (6d, see Brans & De Meester, 2018).

Experimental design

General set-up

Both *Ischnura* and *Daphnia* individuals (or cohorts) were reared at 20 °C or 24 °C and passed through either of two heatwave temperature increases (+4 °C or +8 °C), leading to the following three heatwave treatments (Fig. 1): HW20-28°C inducing a shift from 20 °C (rearing temperature) to 28 °C (heatwave temperature), HW24-28°C, implying a shift from 24 °C (rearing temperature) to 28 °C (heatwave temperature), and HW24-28°C coinciding with a shift from 24 °C (rearing temperature) to 32 °C (heatwave temperature). Individuals coming from 20 °C did not pass through a heatwave of 32 °C as the difference in temperature would have been too high.

Using two rearing temperatures enabled us to compare the impact of thermal acclimatization on performance under a certain heatwave temperature (comparing HW20-28°C with HW24-28°C), whereas applying two final heatwave temperatures (28°C vs. 32 °C) also allowed comparing the effects of increased heatwave intensity (HW24-28°C vs. HW24-32°C). We thus note here that our HW treatment implies a crossing of both rearing/acclimatization temperature and the five-day heatwave temperature. We did not include predation trials performed at the two rearing temperature without heatwave treatments (i.e. 20 °C, 24 °C) as a control. We aimed a testing the effect of urbanization background (and thermal acclimation) on predation rates under thermal stress, rather than the pure effect of a heatwave treatment on predation rates.

Figure 1 depicts the full experimental set-up with all predation trials for each population origin x heatwave combination, which includes a reciprocal crossing of two urbanization levels (rural/urban origin) x 2 species (predator/prey) x 2 rearing temperatures (20 °C or 24 °C) x 2 heatwave treatments

(28 °C or 32 °C), giving a total of 16 combinations. For each combination, 12 replicates were included (total number of experimental units = 144 trials). To maximize the independence of replicates, each replicate consisted of one predator and one prey cohort (20 water fleas), which were combined in such a way that in each treatment combination of 12 replicates, damselfly larvae of an urbanization category originated from different females of the three damselfly populations of origin, and were combined with a different clone of the four *Daphnia* populations. For example in the trials combining urban damselflies with urban water fleas in a certain heatwave treatment (e.g. HW20-28°C), each of three damselfly populations were represented by larvae originating of four different females (n = 3 populations x 4 females= 12 replicates), which were exposed to one clone of each of four *Daphnia* populations, with all 12 clones (n = 4 populations x 3 clones) only used once in the entire block of 12 replicates.

Heatwave treatment and predation trial

Both damselfly larvae and water flea-cohorts passed separately through a five-day heatwave of either 28 or 32 °C according to the specific heatwave treatment (HW20-28°C; HW24-28°C; HW24-32°C). During the heatwave, damselflies were reared in individual 200ml plastic cups, whereas *Daphnia* were reared in 2l plastic aquaria (filled with 1 liter of dechlorinated water). Medium was renewed at the start of the heatwave (inoculation), at day 3, and at the end of the heatwave (day 5, just before the predation trial). Heatwaves were induced in such a way that temperatures gradually increased during the first two days of the heatwave. On day one (before 9 a.m.), aquaria and cups were placed in a temperature controlled water bath, set at the appropriate temperatures (24° C for organisms of HW20-28°C and HW24-28°C; and 28 °C for animals of HW24-32°C). On day two of the heatwave (before 9 a.m.) larvae were moved to their final heatwave temperature. At the end of the heatwave (day 5) the damselfly larva and 20 *Daphnia* individuals were exposed to each other in a 90-minute predation trial (after 2pm), which was recorded on video (Fig. 1).

Prior to performing the predation trials, five randomly chosen sub-adults of the prey species were measured (center of the eye to the base of the tail spine) and 20 (out of 30 water fleas that entered the heatwave) randomly chosen individuals were placed in a new trial aquarium with fresh medium, incubated at the appropriate temperature for 2 hours. In case of *Daphnia* mortality during the heatwave treatment, the specific number of animals transferred to the predation trial aquarium was noted. The number of trials with a number of prey items < 20 is low (i.e. n= 6 out of 144, and all six had between 16 and 19 *Daphnia* prey items at the start of the predation trial).

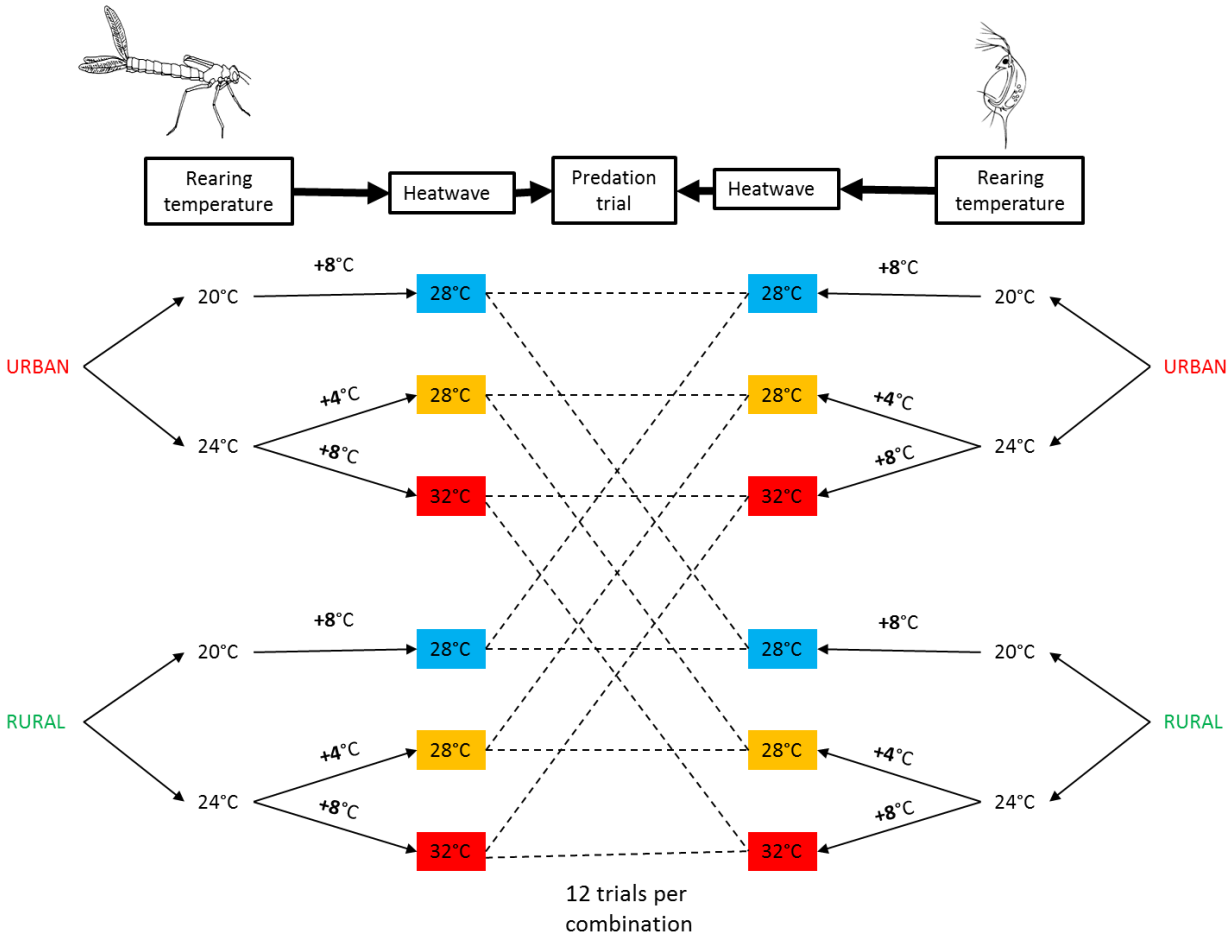

Figure 1. Schematic representation of the experimental set-up. Both urban and rural *Daphnia* prey (right) and *Ischnura* predators (left) were reared at 20 and 24 °C. After culturing the animals for at least two (water fleas) or one (damselflies, from egg to F0) generation in the lab, upon birth (water fleas, < 24h old) or reaching FO (damselflies, max. days after reaching F0 = 8 d) were transferred to either one of three specific heatwave treatments (HW20-28°C, blue; HW24-28°C, yellow; HW24-32°C, red). Heatwaves were simulated using separate temperature controlled water baths, and temperature increases were according to a stepwise increase. At the end of each 5-d heatwave simulation, predator and prey of each urbanization background were combined in a predation trial (one predator with 20 water fleas), according to a reciprocal crossing (urban x urban, urban x rural, rural x urban, rural x rural) at the specific heatwave temperature (according to the HW treatment). For each predator (urban/rural) x prey (urban/rural) x heatwave (HW20-28°C, HW24-28°C, HW24-32°C) trial combination 12 replicates were performed.

Response variables

During the predation trials, *Daphnia* individuals were manually counted every 30 minutes (t30, t60, t90), which enabled to determine predation rates (the number of *Daphnia* eaten/total number of *Daphnia* present, and this at t30, t60, t90). Each predation trial was recorded on video to assess foraging rate and predator activity. Damselflies were scored for CT_{MAX} after the predation trial, using the same procedure as in Op De Beeck *et al.* (2017). CT_{MAX} is a useful proxy to estimate performance under extreme heat stress, and could generate insights in how urban evolution, likely via thermal adaptation of predators, shapes the outcome of the predation trials. For the same reasoning, damselfly body mass was assessed, as well as their development time (from egg to F0), and later analyzed as a response variable to test for

urban adaptation, and additional added as covariates, given that they can influence predation efficiency. For the *Daphnia* clones used here, such data are presented in Brans *et al.* (2017a,b). After CT_{MAX}, damselflies were weighted, sexed, and flash frozen for physiological analysis (total fat, protein, and sugar content; electron transport system activity; lipid peroxidation).

Daphnia survival success is the inverse of the above described damselfly predation rate, which will be used as response variable (see statistical for model structure). *Daphnia* were measured before the predation trial, to verify previously found genetic size differences between urban and rural populations, given that the genotypes used here are a subset of those reported on in Brans *et al.*, 2017a,b. Such size difference might significantly impact predation rates, as smaller prey is easier to catch and involve a reduction in predator handling time. After the predation trials, *Daphnia* were frozen to measure the same stress physiological endpoints as the damselflies.

We here discuss the first part of the results, including *Daphnia* body size, *Ischnura* development time, body mass and CT_{MAX}, and lastly predation rates.

Statistical analyses

All statistical analyses were conducted with R software (R Core Development Team, 2016).

Daphnia *size and* Ischnura *weight (wet mass), development time, and CTmax*

We first separately tested for an effect of urbanization background (categorical; urban vs. rural), HW treatment (categorical; HW20-28°C, HW24-28°C, HW24-32°C), and the combination of both, on the body size of *Daphnia magna*, and the body mass (wet weight) and CT_{MAX} of *Ischnura*. In both cases we applied a linear mixed-effect model (package 'lme4'; Bates *et al.*, 2014; R Core Development Team, 2016) including both fixed effects (urbanization, HW), their interaction, as well as population (nested in urbanization) and clone (for *Daphnia*) or female (for *Ischnura*), nested in population, as random effects. We additionally included date of measurements (heatwave trial) as random effect in both analyses. Given that damselflies were weighted after the predation trial, we assessed *Daphnia* wet weight (calculated using the body size – dry weight conversion equation given in Dumont *et al.*, 1975, and a conversion factor of 6.5 for dry to wet mass) as a covariate to correct for a possible bias driven by a differential amount of *Daphnia* eaten during trials in specific combinations. Additionally for damselflies we included the sex of the individual (male/female) as an additional factor in the analyses (and interactions with HW and/or urbanization). In case of significant HW effects, we applied Tukey post-hoc tests to test for between-heatwave differences.

For *Ishnura* development time we used a similar model structure. Given that this variable measures development from egg stage to F0, HW treatment is replaced with rearing temperature (20 vs. 24 °C) in the model, as development time is independent of HW treatment

Predation rate

To test for an effect of urbanization background of both predator and prey, the HW treatment, and their interactions, we constructed a generalized linear mixed-effect model with a repeated measures structure (t30, t60, t90) and a binomial error distribution (combining number of *Daphnia* eaten and number of *Daphnia* left, given the initial number of *Daphnia* sometimes deviated from the set cohort of 20 individuals). We included *Daphnia* wet mass and damselfly wet mass, corrected for the number of eaten *Daphnia* (damselfly wet mass t0 = damselfly wet mass t90 – number of *Daphnia* eaten x average wet mass per water flea individual in the trial; calculated separately for each trial) as covariates in the analyses.

RESULTS

Ischnura development time and CT_{MAX}

Ischnura development time was significantly longer in urban compared to rural damselflies (χ^2 = 22.019, p = 0.001, Fig. 2) and in animals reared at 20 °C (χ^2 = 6.061, p = 0.004, Fig. 2). Additionally, males developed faster compared to females (χ^2 = 7.728, p = 0. 005, Fig. 2). Analysis of CT_{MAX} showed no difference between urban and rural damselflies, although heatwave treatment significantly increased *Ischnura* heat tolerance (p < 0.001, Table 1).

Ischnura body mass (wet weight) and *Daphnia* body size

The linear mixed-effect models testing the effects of heatwave treatment, urbanization and their interaction on *Ischnura* body mass (Fig. 3) and *Daphnia* body size (Fig. 4) and indicate in both cases a significant effect of urbanization background (predator, p = 0.003; prey, p = 0.001; Table 1) and, for the *Daphnia* prey, a significant heatwave effect (p = 0.004, Table 1). Urban *Ischnura* larvae were heavier compared to rural animals (Fig. 3). Urban *Daphnia* were significantly smaller compared to rural *Daphnia* (Fig. 4). For *Daphnia*, post-hoc tests revealed heatwave treatments HW20-28 differing from HW24-32 (p = 0.017), thus reflecting rearing temperature rather than the heatwave temperature increase (+ 8 °C) drives the plastic size reduction.

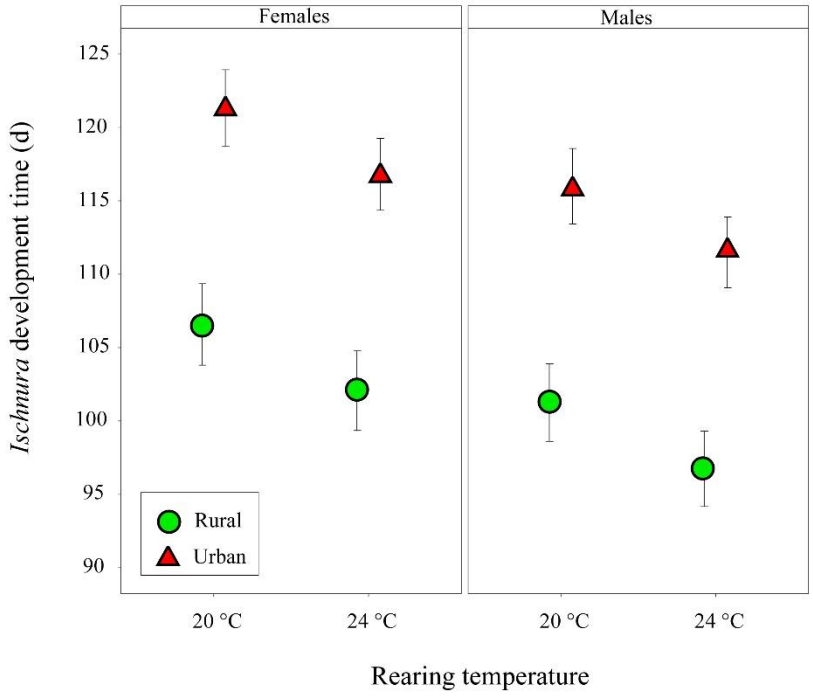

Figure 2. *Ischnura elegans* (predator) development time (days until reaching the F0 larval stage) in response to rearing temperature (20 vs. 24 °C), urbanization class (rural: green circles, urban: red triangles), and sex (females: left panel, males: right panel). The figure shows least-square means ± 1SE.

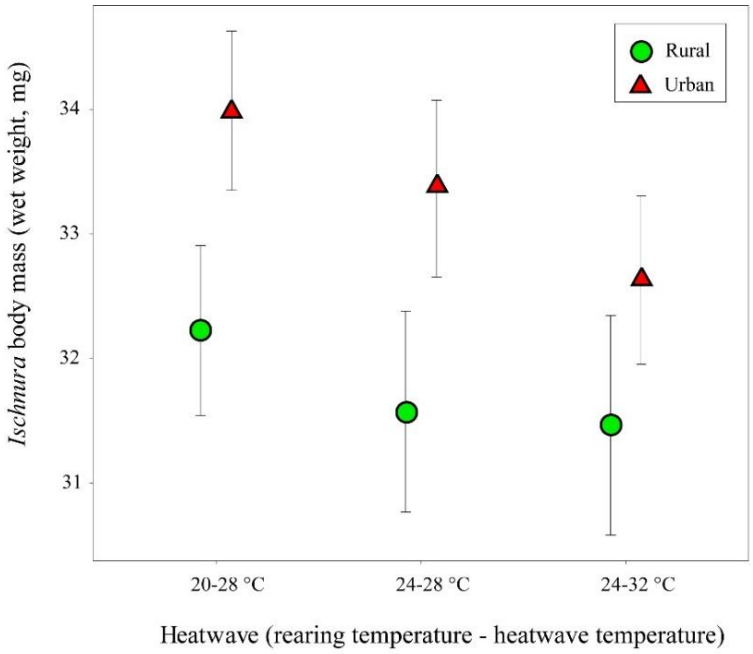

Figure 3. *Ischnura elegans* (predator) body mass (wet weight, mg), in response to heatwave treatment and urbanization level (rural: green circles, urban: red triangles). The figure shows least-square means ± 1SE..

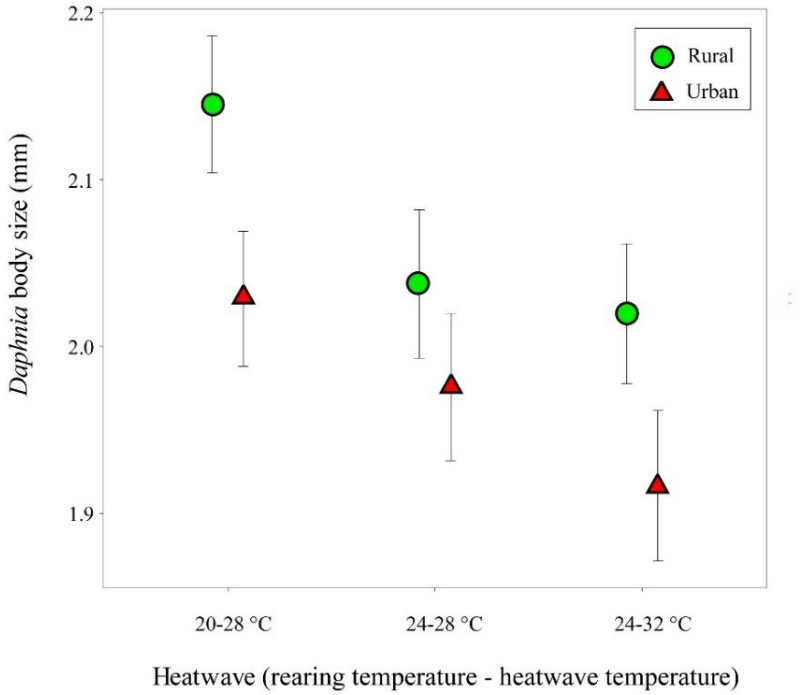

Figure 4. *Daphnia magna* (prey) body size in response to heatwave treatment and urbanization level (rural: green circles, urban: red triangles). The figure shows least-square means ± 1SE.

Predation rate

Figures 5 and 6 show mass-corrected predation rates as given by the number of *Daphnia* eaten. Results show predation rate is significantly impacted by the heatwave treatment ($p = 0.002$, Fig. 5, Table 2) and he urbanization background of the prey and predator (interaction term $p = 0.036$, Fig. 6, Table 2). Post-hoc tests revealed a significant difference between HW20-28°C and HW24-28HW°C ($p = 0.02$, Fig. 5) and HW20-28°C and HW24-32°C ($p = 0.018$, Fig. 5). Additionally prey biomass and time point (t30, t60, t90) significantly contributed to the variation in predation rate (Table 2), with predation rates (cumulative) increasing with time ($p < 0.001$) and increasing with lower prey biomass ($p = 0.005$).

When looking at the significant interaction effect of the urbanization background of predator and prey, we found that urban *Ischnura* larvae have a higher predation rate compared to rural larvae, but only in presence of rural *Daphnia*. When predating on urban *Daphnia*, no difference is found between urban and rural predator larvae (Table 2, Fig. 6).

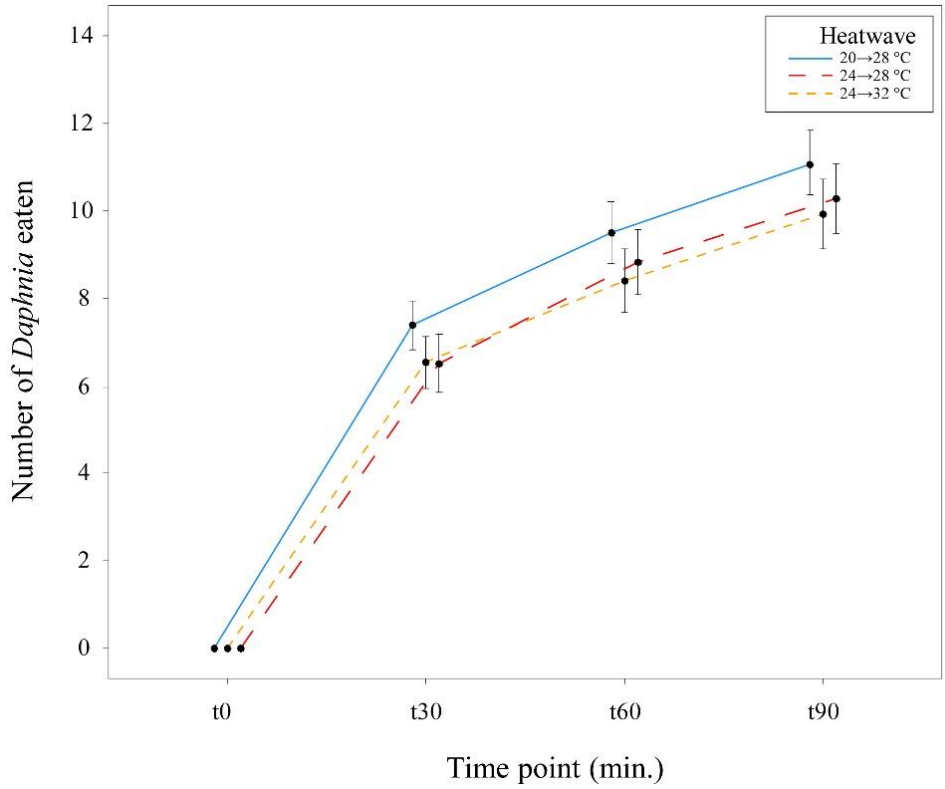

Figure 5. Number of *Daphnia* eaten at each 30-minute time interval and in response to the heatwave treatment (HW20-28°C, blue full line: HW24-28°C, orange dashed line; HW24-32°C, red dashed line). The figure shows least-square means ± 1SE.

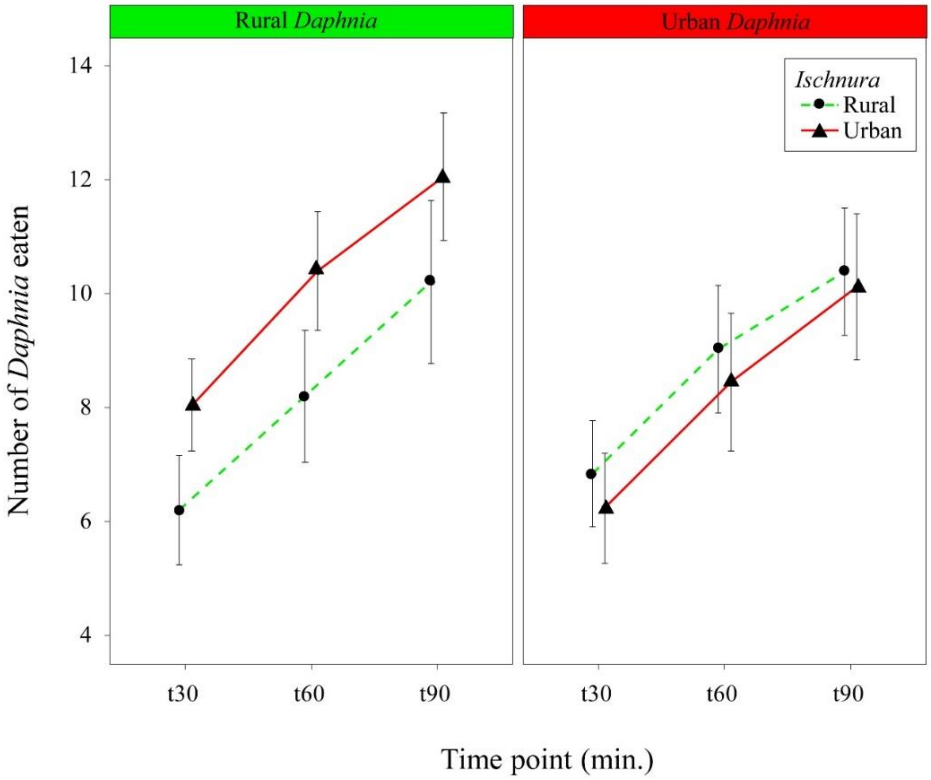

Figure 6. Number of *Daphnia* eaten (cumulative) at each time point (t30, t60, t90) in response to the urbanization level of the *Daphnia* prey (left panel: rural *Daphnia*, right panel: urban *Daphnia*) and *Ischnura* predators (rural

Ischnura: dashed green line, urban *Ischnura*: red full line). The figure shows least-square means ± 1SE, pooled across HW treatments.

Table 1. Linear mixed-effect model results for *Daphnia* size (mm) and *Ischnura* body mass (mg) and CT_{MAX} (°C), in response to heatwave treatment (HW; HW20-28°C, HW24-28°, HW24-32°C), urbanization (urban/rural), and their interaction. For *Ischnura*, sex of the larvae was added as an additional factor (including interactions with HW and urbanization class). For both analyses, clone (*Daphnia*) and female (*Ischnura*, mother female), nested in population, and population, nested in urbanization class, were added as random effects.

	***Daphnia* size (mm)**			***Ischnura* body mass (wet weight, mg)**			***Ischnura* CT_{MAX} (° C)**		
Effect	**Wald χ^2**	**Df**	**p**	**Wald χ^2**	**Df**	**p**	**Wald χ^2**	**Df**	**p**
Heatwave (HW)	10.923	2	**0.004**	1.432	2	0.489	**33.711**	**2**	**< 0.001**
Urbanization	10.379	1	**0.001**	**8.872**	**1**	**0.003**	0.162	1	0.687
Urbanization x HW	0.883	2	0.643	0.104	2	0.949	3.611	2	0.164
Sex				19.139	1	**<0.001**			
Urbanization x sex				0.229	1	0.632			
HW x sex				7.779	2	**0.020**			
Urbanization x HW x sex				0.487	2	0.784			

Table 2. Generalized linear mixed-effect model (repeated measures) results for predation rate in response to heatwave treatment (HW; HW20-28°C, HW24-28°, HW24-32°C), urbanization level (urban/rural) of both *Ischnura* predators and *Daphnia* prey. Prey wet mass and predator wet mass (corrected for number of *Daphnia* eaten) were included as random effects, in addition to the random nested structure of clone (*Daphnia*) or female (*Ischnura*, mother female) nested in population, and population nested in urbanization category.

Predation rate			
Effect	**Wald χ^2**	**Df**	**p**
Heatwave treatment (HW)	12.3614	2	**0.002**
Urbanization prey	4.620	1	**0.032**
Urbanization predator	2.243	1	0.134
HW x urbanization prey	3.019	2	0.221
HW x urbanization predator	0.610	2	0.737
Urbanization predator x urbanization prey	4.379	1	**0.036**
HW x Urbanization predator x urbanization prey	1.467	2	0.480
Prey (*Daphnia*) wet mass (mg)	7.987	1	**0.005**
Predator (*Ischnura*) wet mass (mg, corrected)	0.007	1	0.931
Time	243.028	2	**<0.001**

DISCUSSION

In this study, we set out to test how evolution in response to urbanization in predator (damselfly *Ischnura elegans*) and prey (water flea *Daphnia magna*) species impacts trophic interactions between both organisms. Given the strong temperature-association of the previously reported genetic differentiation in both prey (direct link via increased temperatures in urban settings; Brans *et al.*, 2017a,b, 2018a; Brans & De Meester, 2018) and predator (indirect link via phenological changes associated with longer growing season in urban settings; Tüzün *et al.*, 2017b), we assessed predation rates in simulated heatwaves, as to determine how thermal adaptation in city populations shapes predator-prey interactions under thermal tress. The five-day simulated heatwaves were a combination of a 20/24 °C rearing temperature and + 4 and/or + 8 °C effective heatwave temperature increase. Heatwave treatments affected individual response variables, such as predator CT_{MAX}, which increased with higher rearing temperatures and more extreme temperature exposures. There was a significant impact of heatwave treatment on prey body size, but post-hoc tests revealed that the reduction in body size was mainly driven by the increase in rearing temperature, rather than the effective heatwave exposures. Predator body mass was not affected by heatwave treatment. Higher ambient rearing temperatures and heatwave intensity negatively affected predation rate, with lowest predation rates observed for those animals reared at 24 °C and exposed to +4 °C or +8°C heatwaves. Post--hoc comparisons of heatwave treatments revealed, however, that rather than short-term exposures to intense heatwaves, the increase in ambient rearing temperature (20 °C vs. 24 °C) affected response variables more than the 5-day exposure to extreme heatwave temperatures. For example there was no differentiation between HW24-32°C and HW24-28°C for predation rate.

While urbanization affected both damselfly development time (slower) and body mass (heavier), as well as *Daphnia* body size (smaller), in line with previous findings, even when correcting for these urban-driven differentiation in traits, predation rates were still impacted by urbanization background of both parties. As we did a common garden experiment, purging maternal effects for one (damselflies) or two generations (water fleas), this result implies that evolution of both prey and predator in response to urbanization impacts the outcome of their interaction. The experimental suggest an asymmetric 'mismatch-effect'; predation rates of urban damselflies are higher compared to rural damselflies, but only when they feed on rural prey. Exposing urban predators to urban prey negatively affects predation rates compared to combinations with rural prey. Conversely, when looking at rural predator feeding rates, this mismatch effect is not present (equal survival of both rural and urban *Daphnia*). These result also document a clear case of cryptic eco-evo dynamics; including the evolutionary background of not only the predator, but also the prey, substantially altered the observed predation rates. In the following paragraphs we will detail results on all response variables separately, with a main focus on the effects of urbanization.

The impact of urbanization and temperature stress on predator and prey functional traits

Urban *Daphnia* were significantly smaller then rural *Daphnia*, confirming previously reported results on an extended set of genotypes from the same ponds and study region (Brans *et al.*, 2017a,b). *Ischnura* development time was significantly longer and larvae were heavier at F0 stage in urban settings, which also aligns with previous results on a different damselfly species in the same study region (Tüzün *et al.*, 2017b). In the latter study, Tüzün *et al.* (2017b) showed larvae of *Coenagrion puella* were characterized by a lower growth rate in urban populations when reared along a temperature gradient in the lab (16-20-24-28-30 °C). The earlier onset and prolongation of growing seasons in urban ponds (Brans *et al.*, 2018a) is suggested to reduce the time stress to develop to F0 stage before winter starts in these urban populations, whereas for rural populations, shorter growing seasons imply a shorter thermal window to develop into F0 before hibernation, and thus selection for faster growth and development. While we only assessed performance at two temperatures, the results on *Ischnura elegans* presented here are similar to the previous observations in *Coenagrion*, and indicate urbanization and its associated warming in the study region similarly shape genetic differentiation of functional traits in both species via an indirect phenological countergradient mechanism (Conover & Schultz, 1995; Conover *et al.*, 2009). Urbanization did not impact CT_{MAX} of damselfly larvae, similar to what has been reported for damselflies along larger geographical gradients (Addo-Bediako *et al.*, 2000; Op de Beeck *et al.*, 2017), and in support of a vertical, not horizontal shift in TPC. Vertical shifts in TPCs are more often linked to up- or downregulation of enzyme functioning (Angilletta *et al.*, 2003), whereas horizontal shifts imply radical changes in physiological characteristics and molecule structures (i.e. thermokinetics of enzyme functions; Yamahira *et al.*, 2007; Angilletta, 2009; Berger *et al.*, 2013). Exposures to warmer rearing temperature and heatwave temperatures induced a plastic shift in CT_{MAX}. The latter has been suggested to be often due to transient increases in haemoglobine or heat shock protein concentrations, or DNA-repair upregulation (Pörtner *et al.*, 2017).

Cryptic evo-evo dynamics in the city

By including evolution (urbanization background) in two interacting species, our data show a clear case of cryptic eco-evo dynamics (Kinnison *et al.*, 2015). There is an increasing number of studies showing that evolution of organismal traits can affect ecological processes (Yoshida *et al.*, 2003; Fussmann *et al.*, 2007; Johnson *et al.*, 2009; Alberti *et al.*, 2017b; Hendry, 2017). Here, we show that for two key species of the freshwater aquatic food chain, both showing genetic adaptation in response to urbanization (*Ischnura*; this study; *Daphnia*: Brans *et al.*, 2017a,b and this study) the combined effect of their evolutionary background alters the ecological response (i.e. predation rate) in such manner that evolution in the prey buffers for the evolutionary response of the predator. If only the evolutionary background of one of the two species would be known or included, this effect would not be detected, hence, it is cryptic.

Urban *Daphnia* evolved a higher heat tolerance and increased haemoglobin concentrations (Brans *et al.*, 2017b), a smaller size at maturity (Brans *et al.*, 2017a; and this study), and have higher concentrations of total fat, protein and sugars available (Brans *et al.*, 2018b). All traits render them most likely better equipped to cope with the higher temperatures they are exposed to in the heatwave trials (higher heat tolerance, more efficient oxygen transportation, sufficient energy resource availability). The observed mismatch effect might thus be a result of rural *Daphnia* being less difficult to catch because their performance is impaired at high temperatures. On the other hand, while counterintuitive (based on development time), we hypothesize that urban damselflies might show higher attack rates and predation activity in comparison to rural damselfly larvae to acquire enough resources (see also Therry *et al.*, 2014 for higher larval activity in edge populations). One plausible reason is the higher need of energy reserves as a larvae or in later life stages (adult terrestrial stage) in urban compared to rural environments. Tüzün *et al.*, (2017a) found urban, but not rural, damselflies to be characterized by a behavioral syndrome including activity and boldness, which might indeed be related to an increased food intake in urban ponds. Energy resources can be allocated to stress tolerance mechanisms. These can include tolerance mechanisms to thermal stress in both life stages, given that both terrestrial and freshwater ponds are impacted by the urban heat island effect (Brans *et al.*, 2018a; Wouters *et al.*, 2017), but possibly also tolerance to other urbanization associated environmental changes like pollution or hypoxia (larval stage). In addition, higher concentrations of energy metabolites as larvae might increase adult survival and performance. One possible performance trait in this case is flight endurance. Tüzün *et al.*, (2017c) showed urban damselfly adults to be characterized by a higher flight performance compared to rural adults, which could be a result of an adaptive carry-over effect linked to energy acquisition in the larval stage. In conclusion, the combined effect of higher energy acquisition in urban compared to rural damselflies (possibly linked to adult flight endurance, although not explicitly tested in this study) and the higher heat tolerance and performance under thermal stress in urban compared to rural water flies, likely resulted in the observed cryptic eco-evolutionary response in predation rate.

The results of this study, in combination by the observed higher flight endurance in urban damselflies can be highly relevant in scenarios of range expansion out of the city. Damselflies show indications of spatial sorting across both larger and smaller geographical distances (Swaegers *et al.*, 2015; Tüzün *et al.*, 2017c). Damselfly populations are hypothesized to undergo spatial sorting from the rural to the urban city centers. Higher habitat fragmentation with increasing urbanization selects for higher dispersal capacity and, consequently, only the best flyers reach the urban ponds, which results in typical core-edge dynamics. Nevertheless, these dynamics might eventually result in a reverse pattern of spatial sorting (especially in cities with distinct urban cores), namely dispersal in the urban-to-rural direction, and thus out of the city. After mating and egg-deposition in novel ponds in suburban and rural areas, their intrinsically differently evolved developmental (Tüzün *et al.*, 2017b; and this study) and performance traits (activity and boldness, Tüzün *et al.*, 2017a; predation, this study) might result in

being superior predators upon encounter of suburban and rural water flea populations during summer. Thus, the observed cryptic eco-evo dynamics can result in profound changes in food web structure when both species encounter each other in a novel context.

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General Discussion

1 Urban hot-tubs and multi-trait adaptation in *Daphnia magna*

The results of our common garden experiments outlined in Chapter 2-4 indicate genetic differentiation between urban and rural *Daphnia magna* populations for a wide set of functional traits, involving multiple life-history, energy storage and stress physiological response traits. The majority of the observed responses align with the hypothesis that urbanization-driven warming elicits genetic differentiation for functional traits such as heat tolerance and life histories (Hypothesis 1 and 2). In this section, I will first (paragraph 1.1) discuss the observed genetic trait shifts in the light of the observed thermal characteristics of urban compared to rural ponds in the study area. In paragraph 1.2 I will address potential alternative selection pressures that might explain the observed responses. In paragraph 1.3 I will briefly discuss the alternative explanation that the observed trait shifts might have resulted as a consequence of neutral evolution. The results of this thesis are conceptualized in Figure 1.

1.1 Adaptive evolution in response to warming

1.1.1 Evolution of mean trait values

Heat tolerance

The results of Chapter 1 suggest that urban ponds provide a differential thermal selective environment compared to rural ponds, mainly driven by a substantial difference in mean and maximum temperatures, as well as in temperature variability and in length of the growing season. These results confirm Hypothesis 1 ("*Urbanization, via the urban heat island effect, impacts the thermal environment of small- to intermediate-sized closed aquatic ecosystems such as freshwater ponds and small lakes*)*".* During the summer season urban ponds were characterized by temperature increases of 3.2 °C (i.e. daily mean temperature) and 3.7 °C (i.e. daily maximum temperature) compared to rural ponds. The observed evolved higher heat tolerance in urban populations (Chapter 2) is most likely a direct adaptive response to this 'urban hot-tub' effect that we observed for the study region (Flanders, Belgium; Chapter 1) and parallels terrestrial studies reporting on urban thermal adaptation (i.e. in CT_{MAX} responses) in response to urban heat islands (e.g. Diamond *et al.*, 2017b). Our results on the increase in heat tolerance and haemoglobin concentration in urban compared to rural *D. magna* population are in line with Hypothesis 2 ("*Compared to rural populations of* D. magna*, urban populations show genetic differentiation in life history and stress physiology, reflecting adaptive evolution to warming*").

While the increase in heat tolerance aligns with the increase in mean temperatures in urban compared to rural ponds, it is equally or even more likely that CT_{MAX} evolved in response to higher maximum and extreme temperatures or increased temperature variability in urban compared to rural ponds (Bozinovic *et al.*, 2011; Clusella-Trullas *et al.*, 2011; Geerts *et al.*, 2015; Bukcley & Huey, 2016). This would be in line with the Climate Variability Hypothesis (CHV; Ghalambor *et al.*, 2006; Gutiérrez-

Pesquera *et al.*, 2016; Shah *et al.*, 2017). Jørgensen *et al.* (unpublished data and personal communication) found a clear link between the maximum temperature of the warmest month and precipitation levels in the driest months with among-population differences in CT_{MAX} in *Drosophila*. Based on common garden experiments, Diamond *et al.* (2017b, 2018b) reported higher CT_{MAX} in urban compared to rural acorn ant (*Temnothorax curvispinosus*) populations. In the set of ponds that we monitored for temperature in Chapter 1, we measured mean daily temperatures up to 34.2 °C (averaged over a 24h period), with a daily temperature range of 3.5 °C. Such temperature extremes impair growth and reproduction, and a higher heat tolerance can be crucial for population survival during heatwave periods in urban ponds. Such heatwave periods thus likely cause strong selection for genotypes with a high heat tolerance (Overgaard *et al.*, 2014; Diamond *et al.*, 2017b; 2018a,b; Geerts *et al.*, 2015). When conditions shift back to temperatures close to the optimal temperature for growth and reproduction, given by the peak of the thermal performance curve (Conover & Schultz, 1995; Conover *et al.*, 2009; see also Fig. 3), growth and reproduction can resume (Overgaard *et al.*, 2014).

While criticized for its relevance as a performance trait (e.g. Lutterschmidt & Hutchison, 1997; Santos *et al.*, 2011) other studies (Overgaard *et al.*, 2014; Buckley & Huey, 2016; Kinsgolver & Buckley, 2017; Pörtner *et al.*, 2017) indicate that CT_{MAX} as a tolerance characteristic can be a crucial indicator of population survival under extremes. In our study system, CT_{MAX} might be highly relevant, as urbanization coincides with a higher frequency of extreme temperature events (De Ridder *et al.*, 2015; Wouters *et al.*, 2017; Diamond *et al.*, 2018b). In the context of species range distributions, several studies show that critical thermal values such as CT_{MAX} and CT_{MIN}, and thermal breadth (i.e. the difference between both critical thermal values) are more successful in explaining occupancy ranges across climatic gradients than thermal performance curves (Deutsch *et al.*, 2008; Sunday *et al.*, 2011; Overgaard *et al.*, 2014). This confirms the importance of such tolerance limits in ecological and evolutionary studies (Terblanche *et al.*, 2011; Overgaard *et al.*, 2012; Baudier *et al.*, 2018; Jørgensen *et al.*, unpublished data).

Life history and stress physiology

Our data on life-history and stress physiological traits align with the hypothesis of adaptation to an increase in temperatures in urban ponds (Chapter 2,3,5). We found an evolutionary reduction in size at maturity in urban compared to rural *D. magna* populations as well as an overall faster multivariate pace-of-life (Fig. 1). While the genetic differentiation in multivariate life-history trait space was smaller than observed phenotypic plasticity responses to warming (i.e. measurements at 20 vs 24 °C; see Phenotypic Trajectory Analysis in Chapter 3), both genetic (evolutionary) and plastic (thermal plasticity) trajectories were directionally similar (i.e. along the PC1 axis, coinciding with fast development, high fecundity, and high intrinsic population growth rates; Chapter 3). The latter supports the suggestion that the drivers of genetic differentiation might be linked to warming.

The evolution of a smaller size at maturity is in line with the widely studied Bergmann's rule (James, 1970; Pincheira-Donoso *et al.*, 2008; Shelomi, 2012; Teplitsky & Millien, 2014) and the temperature-size rule in ectotherms (TSR; Aktinson, 1994; Arendt, 2009; Horne *et al.*, 2016). Both concepts puzzled researchers for decades (Atkinson, 1994; Angilletta & Dunham, 2003; Walters & Hassall, 2006; Klok & Harrison, 2013; Hoefnagel *et al.*, 2018). The temperature-size rule states that ectotherm species grow faster but have a smaller size at maturity under warmer conditions. This pattern is widely observed (Atkinson 1994), but some studies have reported exceptions (e.g. Walters & Hassall, 2006, for the grasshopper *Chorthippus brunneus*). Such patterns may arise from increased developmental rates relative to growth rates at higher temperatures (Forster & Hirst, 2012). Alternatively, resource limitation can more strongly impact larger individuals at higher temperature (Edeline *et al.*, 2013), or the pattern might reflect that smaller organisms are more efficient in oxygen transport due to their larger surface-area-to-volume ratio in comparison with larger organisms. Maturing at a smaller size diminishes respiratory stress and thus possibly oxidative damage at higher temperatures (Seidl *et al.*, 2005; Angilletta, 2009; Hoefnagel *et al.*, 2015, 2018; Horne *et al.*, 2016). The temperature-size rule has been intensively studied across latitudinal, altitudinal, and seasonal (Horne *et al.*, 2015; Horne *et al.*, 2016; Horne *et al.*, 2017) temperature gradients in terrestrial, marine, and freshwater ectotherms. It has been reported that the pattern is strongest in freshwater organisms, likely because of warming-induced hypoxia in these systems (Forster *et al.*, 2012; Horne *et al.*, 2015; Verberk *et al.*, 2016). The combination of an increased metabolic rate and a decreased oxygen solubility in water upon warming results in a strong change in ratio between oxygen demand and supply with increasing temperatures in aquatic ecosystems. This might promote fast juvenile development and maturation at smaller size. This size reduction decreases respiratory stress and enables efficient oxygen transportation through the body (Hoefnagel *et al.*, 2018).

In line with the hypothesis that thermal stress in urban ponds might also be associated with hypoxia, we observed that urban compared to rural *Daphnia* populations show a significant increase in haemoglobin concentrations (Chapter 2). While we did not compare performance of urban and rural animals under normoxic and anoxic conditions, the observed genetic differentiation in haemoglobin content between urban and rural *Daphnia* likely reflects the evolution of higher hypoxia-tolerance (Pirow *et al.*, 2001; Gorr *et al.*, 2004; Paul *et al.*, 2004). Hypoxia and cyclical hypoxia have been shown to feed back to heat tolerance and life-history evolution via the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis in both terrestrial and aquatic organisms (Pörtner & Knust, 2007; Pörtner & Farrell, 2008; Pörtner, 2012; Giomi *et al.*, 2013; Smith *et al.*, 2015; Shea *et al.*, 2016; Pörtner *et al.*, 2017; Teague *et al.*, 2017). The OCLTT hypothesis suggests that the mismatch between the demand for oxygen and its supply to the tissues is one of the first functional mechanisms determining the aerobic scope and whole-organism tolerance to extreme temperatures. There is quite some empirical support for the OCLTT hypothesis in many studies on open water organisms exposed to gradual temperature

changes (e.g. Frederich & Pörtner, 2000; & Pörtner & Knust, 2007). The OCLTT hypothesis might also be relevant to responses to diurnal temperature and O_2 fluctuations. For example, Peruzza *et al.* (2017) recently reported that exposure to diurnal cyclic hypoxia in the intertidal decapod crustacean *Palaemon varians* can impair individual growth rates, but also increases thermal tolerance and copper tolerance through changes in gill morphology. Because of the combined effect of their high productivity and the urban heat island effect, such diurnal variations in oxygen concentrations and regular or occasional hypoxia might also occur in small urban ponds during warm periods (Heiskary, 2001; Paul & Meyer, 2001; Mallin *et al.*, 2006; Spinetti, 2012). A typical diurnal oxygen cycle is sinusoidal with the highest oxygen concentrations late in the afternoon and lowest early in the morning. Eutrophication-driven increases in productivity might strongly increase the amplitude of these fluctuations, resulting in supersaturation in oxygen during the day but very low levels during the night due to the combined effect of respiration by the high densities of algae and the oxygen consumption linked to microbial degradation of organic material. It is likely that the evolution of higher haemoglobin concentrations that we observe is linked to such diel hypoxia oscillations. Such a response to hypoxia can feed back to heat tolerance, as it selects for more hypoxia-tolerant genotypes and thus increases OCLTT (Paul *et al.*, 2004: Williams *et al.*, 2011; Cambronero *et al.*, 2018). This feedback was, however, not confirmed by our path analysis (Chapter 2), as we found no evidence that the higher haemoglobin concentrations per unit of dry weight in urban compared to rural populations increased their heat tolerance. The higher haemoglobin concentrations still suggest evolved hypoxia-tolerance in urban populations, adaptive at higher temperatures and low oxygen concentrations. The recently published resurrection ecology study by Cambronero *et al.* (2018), reporting on an increase in hypoxia-tolerance through a period of increased occurrences of heatwaves and average warming in a lake in Denmark, similarly is suggestive of a close link between warming, heat tolerance, and hypoxia tolerance in *Daphnia*.

In addition to haemoglobin concentration, we also quantified energetic reserve compounds and oxidative stress responses in urban and rural *Daphnia*. Lipids, sugars, and proteins are the biomolecular energetic reserves that are essential for basal metabolic demands and provide the scope to respond to stress. The energetic reserves determine the aerobic scope of an organism, which is defined as the energy flux left to invest in fitness-related processes such as development, growth, fecundity, dispersal and stress responses (De Coen & Jansen, 1997, 2003; Sokolova, 2013), after basic metabolic needs are met. Warming can impair the aerobic scope of organisms due to increased energy demands for cellular protection and repair mechanisms (Lannig & Sokolova, 2008; Vergauwen *et al.*, 2010; Sokolova *et al.*, 2012). This can lead to a reduction in energy allocation to growth, development, and reproduction. This link between energy allocation and stress tolerance was synthesized in the concept of energy-limited stress tolerance (De Coen & Jansen, 1997, 2003; Sokolova & Lannig, 2008; Sokolva *et al.*, 2012) and complements the OCLTT (Pörtner, 2012; Sokolova, 2013). In *Daphnia*, energy molecules are often depleted upon temporary or short-term exposure to temperature stress (Bergman Filho *et al.*, 2011;

Zhang *et al.*, 2016). Our data show that exposure to higher rearing temperatures indeed elicits a plastic reduction in protein content in both urban and rural populations of *Daphnia*. Yet, the results of Chapter 4 also indicate a profound genetic differentiation towards a higher concentration of all energy storage molecules in urban compared to rural populations. This higher amount of energy storage molecules is not observed when expressed per unit of dry mass, but also when expressed per individual. The latter is even more striking, given that urban animals are smaller than rural ones. Given that the animals were reared under optimal food conditions, such genetic trait differentiation for a higher amount of energy compounds could reflect a countergradient response towards higher energy assimilation and storage efficiency under beneficial food conditions linked to selection for an increased scope to deal with high temperatures and other stressors (Pajk *et al.*, 2012). The higher concentrations of fat, protein, and sugars can then buffer cellular repair responses under environmental stress while still maintaining a positive aerobic scope (Sokolova, 2013).

The results that we reported upon in Chapter 4 indicate a more efficient stress tolerance mechanism in urban compared to rural populations. Lipid peroxidation was not significantly differing between the two sets of populations, even though glutathione-S-transferase (GST) and superoxide dismutase (SOD) showed lower constitutive activity in urban compared to rural populations. Urban populations might have adapted to the higher overall stress levels in urban settings by evolving a higher treshold point of activation in these enzymes, thus more efficiently using energy for fine-tuned stress defense mechanisms. This might also be linked to an evolution of the enzymes themselves towards a more efficient physiological operation mode, such as for example reported by Medina *et al.* (2007) for damselflies. Alternatively, it might be that the urban populations evolved other defense mechanisms so that they rely less strongly on oxidative stress responses. One such defense pathway is the production of Heat Shock Proteins (HSP; e.g. Feder & Hofmann, 1999; Pauwels *et al.*, 2007). HSP production is induced under a wide variety of stress conditions such as increased predation, warming and toxicant exposure (Pauwels *et al.*, 2007; Oksala *et al.*, 2014; Ravaschiere *et al.*, 2017). Evolution of higher constitute levels of HSP in *Daphnia* has been reported before (Pauwels *et al.* 2007) and might generate an overall protective cellular mechanism increasing their stress tolerance and reducing their reliance on oxidative stress responses. Interestingly, HSPs can positively feedback to heat tolerance (i.e. $CT_{MAX,}$ Pörtner *et al.*, 2017).

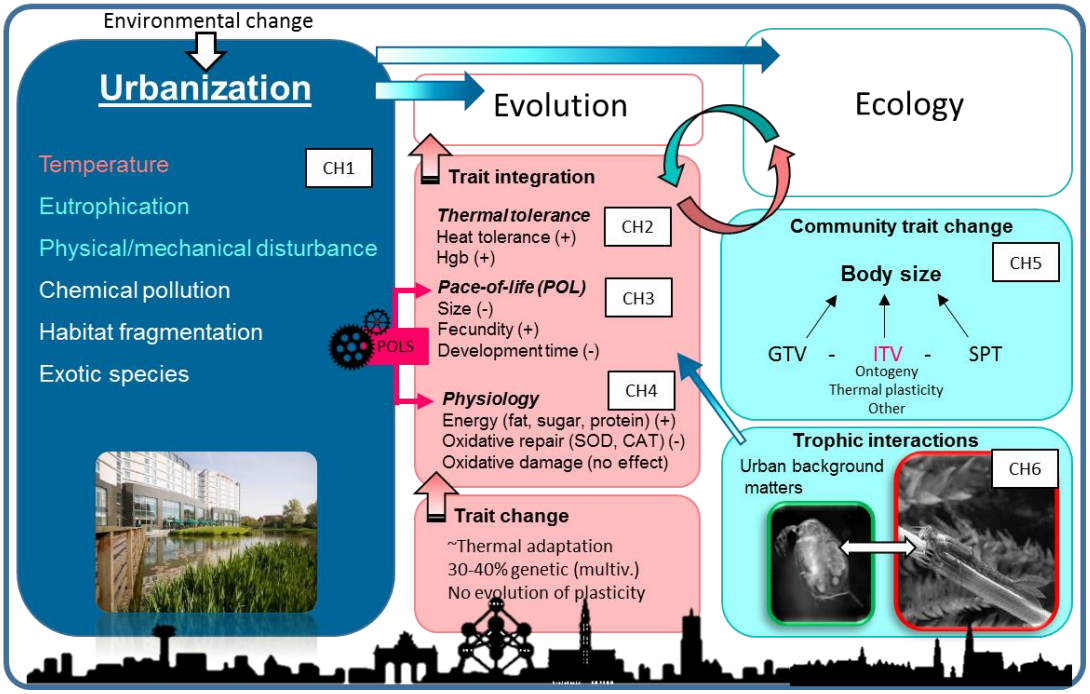

Figure 1. Synoptic integration of the main results presented in Chapters 1 to 6 in the scheme presenting knowledge gaps in urban evolutionary ecology as outlined in Figure 3 of the Introduction.

1.1.2 Evolution of mean trait values and trait integration: the bigger picture

Figure 1 presents the overarching scheme given in the Introduction of this thesis, with a synthesis of the results on mean trait values reported on in Chapters 1-4 (and results of Chapter 5 and 6, see 2. Urban eco-evolutionary dynamics). We showed that the mechanistic link between physiology and life history trait covariation that is indicated by a pace-of-life syndrome (POLS) is present in the urban but not in the rural populations (Chapter 4). A POLS is suggested to structure trait covariation patterns within and between life history, physiology, and behavior along the slow-to-fast pace-of-life axis (Ricklefs & Wikelski, 2002; Réale *et al.*, 2010; Damhahn *et al.*, 2018). Here we present possible mechanisms by which the POLS evolved in the urban *D. magna* populations and how it may support the evolution of a faster pace-of-life.

We hypothesize that urban populations, due to their exposure to higher mean, minimum and maximum temperatures, higher temperature variability, and warming-associated hypoxia, are selected for a higher heat tolerance and smaller body size (Atkinson, 1994). Due to an increase in metabolic rate and cellular processes with increasing temperatures, warming additionally results in a fast development and a faster pace-of-life. The evolution of other life history traits, such as an increased allocation to reproduction and higher population growth rate, contribute to shaping the pace-of-life pattern. The evolution of higher concentrations of energy storage molecules (fat, sugars, protein) in urban compared to rural populations, together with the observed presence of a POLS, tightly linking physiology and life history, likely enables the change in life history towards faster development and population growth in

the urban *D. magna* populations. The evolution of the pace-of-life syndrome in urban *D. magna* thus acts as a 'physiological gearbox' allowing the shift to a faster life style.

The POLS hypothesis recently received criticism due to its limited empirical support, mainly as a consequence of methodological issues in behavioral and personality studies (Damhahn *et al.*, 2018; Montiglio *et al.*, 2018). Our results are in line with the hypothesis that POLS are expected to evolve in highly dynamic environments (Wright *et al.*, in press) and under stressful conditions (Bell, 2005; Sih *et al.*, 2004; Bell & Sih, 2007; Sih *et al.*, 2012; Snekser *et al.*, 2009). We hypothesize that selection pressures in rural ponds substantially differ from the selection pressures present in urban ponds. We did not find evidence for an integrated POLS in rural populations. This might reflect that energy and stress physiology were not selected to align with life history because of a weaker selection for a fast pace-of-life. Trait correlations in rural populations might potentially be stronger between life history, behavior and morphology (anti-predator defense) and immune-defense (Tieleman, 2018). We do not have strong data on the amount of predators or parasites in urban compared to rural ponds, but it might be that the trade-offs in rural settings are shifted towards, for instance, a higher investment in immune or predator defense. Even if abundances of parasites and mutualists would not differ among urban and rural populations, it has, for example, been suggested that host-parasite and host-symbiont interaction strengths might decrease upon warming (Blanford *et al.*, 2002; Hance *et al.*, 2007; Furlong & Zalucki, 2017; Corbin *et al.*, 2017) due to the difference in T_{OPT} in parasites and hosts. If T_{OPT} is lower in parasites, they would be more vulnerable to warming. Future research on, for example, immunological responses in urban versus rural *Daphnia* populations, might elucidate the bigger picture on the underlying mechanisms and trade-offs of trait integration in both urban and rural populations.

Overall, our results suggest evolved stress tolerance in urban populations as a consequence of strong differential selection to allow *D. magna* individuals to persist during more extreme disturbances (e.g. extreme temperature events).

1.1.3 Evolution, plasticity, and the evolution of plasticity

Widely debated over the past few years is the effective role of evolution, plasticity, and the evolution of plasticity to phenotypic trait change observed along different gradients (see e.g. Ancel, 2000; Price *et al.*, 2003; Grether, 2005; Ghalambor *et al.*, 2007; Paenke *et al.*, 2007; Lande, 2009; Merilä & Hendry, 2014; Cameron *et al.*, 2015; Govaert *et al.*, 2016; Stoks *et al.*, 2016). In the light of climate change, it has been argued that plasticity is crucial for short-term population survival and acts as an initial buffer directing phenotypic trait distributions towards an optimum (West-Eberhard, 2003; Stillman, 2003; Gienapp *et al.*, 2008; Hofmann & Todgham, 2010; Boutin & Lane, 2014; Crozier & Hutchings, 2014; Merilä & Hendry, 2014). While it is often claimed that phenotypic plasticity likely is more important than evolutionary change, empirical results are mixed, and rather indicate that both processes are not mutually exclusive and are often equally important (Hendry & Kinnison, 1999; Kinnison & Hendry,

2001; Stockwell *et al.*, 2003; Bradshaw & Holzapfel, 2006; Parmesan, 2006; Ghalambor *et al.*, 2007; Kovach-Or & Fussmann, 2013; Franks *et al.*, 2014; Schilthuizen & Kellermann, 2014; Stoks *et al.*, 2014, 2016; Geerts *et al.*, 2015; Gao *et al.*, 2018). Overall, this is what has been found in the framework of responses to urbanization (Alberti, 2005; Alberti *et al.*, 2017b; Donihue & Lambert, 2015; Johnson & Munshi-South, 2017), and the data we report upon in this thesis corroborate the hypothesis that both phenotypic plasticity and evolutionary trait change contribute significantly to the observed trait shifts along urbanization gradients. Our data on CT_{MAX}, as well as multivariate life-history traits in addition, propose that phenotypic plasticity responses are strikingly similar across urban and rural populations. Our data thus suggest a limited role of evolution of phenotypic plasticity for the traits studied along the urbanization gradient. This does not imply that phenotypic plasticity would not be important, as we observe quite striking phenotypic plasticity responses in all populations. Rather, it might indicate that there is optimal amount of phenotypic plasticity for the traits studied in relation to the variation in, for example, temperatures encountered in both rural and urban ponds. While the contribution of genetic differentiation to trait shifts was often smaller than that of phenotypic plasticity, evolutionary trait change contributed up to maximum 40% (Chapter 2, Chapter 3) to the total phenotypic trait shift observed from rural populations reared at 20 °C to urban populations reared at 24 °C (i.e. cultured at the average summer temperatures of the respective habitats). In the field, phenotypic trait values may obviously be determined by many different factors, including age distribution (see Chapter 5). However, our observation points to the importance of evolution to trait change in response to temperature, which is likely one of the key recurrent environmental gradients linked to urbanization that impacts biota in small standing waters. Especially during extreme events such as heat waves, acclimatization from a background heat tolerance typical for rural populations might not suffice, driving the evolution of a change in mean trait values (Hoffmann *et al.*, 2012; Gunderson & Stillman, 2015; Diamond, 2017; Heerwaarden *et al.*, 2016).

We could not detect a significant evolution of plasticity for the majority of traits (all except size at maturity). Trait versatility can be important and possibly under selection in dynamic environments (De Jong, 2005; Lande, 2009; Chevin *et al.*, 2010; Crispo *et al.*, 2010; Matesanz *et al.*, 2010; Chevin & Hoffmann, 2017; Gibbin *et al.*, 2017; Diamond *et al.*, 2018b). Our data do not show such evolution of plasticity in response to urbanization, even though evolution of plasticity has been found to be important in response to other environmental challenges (e.g. predation pressure, Stoks *et al.* 2016). This may reflect the fact that the variability in temperature regimes in urban and rural ponds is not fundamentally different. The observed evolution of plasticity for size at maturity in urban populations reflects a reduction in plasticity upon warming. This might reflect that urban populations already reached their intrinsic lower body size limits, reducing the scope for further reduction in body size upon warming.

1.2 Alternative drivers of adaptive urban evolution in the water flea *Daphnia*

When considering the observed evolutionary trait shifts in heat tolerance, life history (e.g. size, pace-of-life) and physiological traits (e.g. energy budget), our results align well with the hypothesis of thermal adaptation to the urban heat-island effect (i.e. confirming Hypothesis 1 and 2 outlined in the Introduction). For example, the path analysis that we carried out in Chapter 2 clearly indicated a direct link between urbanization and the evolution of heat tolerance in our analysis that involved multiple cities. The evolution of life-history traits and physiology in response to urbanization can also be interpreted in the context of warming, as we outlined in the previous paragraphs. Yet, we should not ignore that urbanization gradients integrate multiple and potentially interacting selection factors (e.g. eutrophication, mechanical disturbance, pollution, differences in abundance and activity of predators, parasites and mutualists, …) that might contribute to shaping the evolutionary responses to urbanization. These other selection gradients might interact with the response to temperature, or might independently lead to similar or alternative evolutionary trajectories. As an example of the former, the evolution of a higher availability of energy reserves and increased fecundity might not only depend on the thermal environment, but might also be critically dependent on the availability of resources (e.g. primary productivity). As an example of the latter, the evolution of a faster pace-of-life might also be driven by a change in the abundance or activity of predators (Reznick et al 2002; Stoks *et al.* 2016) or by an overall increase in disturbances likely associated with urbanization. In the next paragraphs, we elaborate on the possibility that other environmental gradients influenced the observed evolutionary responses of *D. magna* along the urbanization gradient.

Physical and mechanical disturbance levels are likely higher in urban than in rural ponds, amongst others because of active management of the water level, occasional or regular cleaning of the sludge and litter layers, or sudden (illegal or accidental) discharges of pollutants (Hassall *et al.*, 2014; and personal observations for the studied ponds during subsequent years 2013-2015). While a survey of more than 80 ponds along urbanization gradients in Flanders did not reveal evidence of higher levels of chemical pollution (e.g. heavy metals and pesticides) in urban compared to rural ponds (see Introduction to this thesis and Engelen, 2017) these data need to be interpreted with caution as they involve only one snapshot survey. Recent data on bacterioplankton metagenomics (Mukherjee *et al.*, unpublished) revealed genes associated with various xenobiotic degradation pathways to be enriched in urban compared to rural ponds. Enriched pathways included degradation of atrazine, dichlorodiphenyltrichloroethane (DTT), hexachlorocyclohexane (HCH), styrene, poly-aromatic hydrocarbons (PAHS), caprolactam, and various medical drugs. This points to higher loads of a number of chemicals and of plastics in urban compared to rural ponds. Other studies have pointed to the presence of higher levels of pollution by plastics, road salts, pesticides, and other chemicals entering the system via surface run-off in urban compared to rural waters (Paul & Meyer, 2001; Beasly & Kneale, 2002; Wenger *et al.*, 2009; van Meter *et al.*, 2014; Wang *et al.*, 2016) .

Differences in biotic interactions between urban and rural ponds can also contribute to differential selection. Eutrophication in combination with the urban heat island effect can promote algal and toxic cyanobacteria blooms (Kosten *et al.*, 2012 ; Paerl & Paul, 2012; Waajen *et al.*, 2014; Huisman *et al.*, 2018;). In parallel, higher temperatures might elicit an increased activity of invertebrate predators (Stoks *et al.*, 2012; Stoks *et al.*, 2017). The disturbances linked to toxic blooms and the increased activity of predators might favor a faster life style (i.e. fast development, high alertness, fast reproduction and high fecundity). In conclusion, the observed evolutionary changes in life history and physiology that we observed might be linked to differences in temperature alone, to differences in a multitude of abiotic and biotic stress factors and disturbances, or to a combination of both. We cannot be sure that the only driving factor is the difference in temperature regimes between urban and rural ponds. Given that some of the predictions for evolution in response to warming and in response to an increase in disturbances are similar, the observed evolution might have resulted from the joint effect of multiple gradients linked to urbanization. Given that we also observed a pronounced evolutionary trait shift for heat tolerance, however, it is very likely that temperature is at least one of the important selection factors.

In order for a faster pace-of-life to evolve, food quantity and quality should be sufficiently high (e.g. Peretyatko *et al.*, 2006, 2007; Waaijen *et al.*, 2014, 2016). The higher concentrations of fat, protein, and sugars in the body tissue combined with a faster pace-of-life of our urban compared to rural *Daphnia* populations (Chapter 4) can indeed only be achieved when food sources are of high quality (Rinke & Petzoldt, 2003). A number of studies that integrated the analysis of effects of phosphor limitation to warming indicated that warming can increase phosphor limitation unless it is accompanied with eutrophication (DeMott & Pape, 2005; Seidendorf *et al.*, 2010; Persson *et al.*, 2011; Wojewodzic *et al.*, 2011; Wolinski *et al.*, 2016). We did not see a significant increase in nutrients or algae densities (chlorophyll a) in urban compared to rural ponds in a survey involving more than 80 ponds (Engelen, 2017). Yet, this result needs to be interpreted with caution, as algal biomass is the result of both bottom-up and top-down effects, and chlorophyll a does not provide information on the quality of the food.

1.3 Neutral dynamics and evolutionary clines

The results that I present in this thesis in general align very well with the hypothesis of adaptive evolution in response to urbanization, linked to the urban heat island effect and potentially also to higher levels of mechanical, chemical and biotic disturbances associated with urbanization. Yet, evolutionary clines along disturbance gradients do not necessarily result from responses to natural selection. Evolutionary clines, which are defined as a change of the frequency of heritable phenotypic traits or genotypes along geographical ranges (Huxley, 1938; Endler, 1986, 1977), can covary with environmental gradients even if they are a consequence of neutral dynamics (Vasemägi, 2006; Santangelo *et al.*, 2018). Neutral dynamics include genetic drift and gene flow. Such neutral dynamics

might result in parallel clines depending on the genetic architecture of a trait. When phenotypic traits have a non-additive genetic basis, for example if a substantial amount of trait variation is explained by epistatic interactions, stochastic drift can result in a directional change in allele-frequencies across independent populations, resulting in parallel clines. This is due to the fact that allele frequency changes at a such loci have a disproportional effect on the phenotypic distribution in a population. Urbanization is often strongly linked to habitat fragmentation. Landscape fragmentation can increase levels of genetic drift due to a reduction in population sizes, and reduced gene flow as dispersal rates decline with increasing habitat isolation. Santangelo *et al.*, (2018) recently revisited the empirical data from Thompson *et al.*, (2016), in order to assess the likelihood that the observed parallel clines in cyanogenesis (i.e. the production of hydrogen cyanide – HCN; a common anti-predator defense) in white clover (*Trifolium repens*) along urbanization gradients could be driven by genetic drift. Urban plant populations show a reduction in HCN+ phenotypes, which was suggested to be driven by winter temperatures. Due to higher snow-cover in rural areas, plants are relatively well buffered against cold 'peaks' and frost, which reduces selection against HCN (rupture of the tissue can cause HCN-mediated damage to the plants). HCN expression is controlled by two independently segregating Mendelian loci, for which plants require dominant alleles at both loci in order to produce HCN (i.e. HCN+ phenotypes). Model simulations indicated that neutral dynamics could generate similar clines as observed in the field across the selection gradient. These results indicate that it is important to consider both selection gradients and potential neutral drivers of evolution along urbanization gradients, as both can significantly drive the evolution of parallel clines. Santangelo *et al.* (2018) also showed that gene flow significantly weakened the occurrence of drift-mediated positive clines along the studied gradients. Based on their model results, Santangelo *et al.*, (2018) conclude that neutral dynamics structuring evolutionary clines can occur along environmental gradients, but that the strength, direction, and stability of clines is highly dependent on population sizes along the gradient, the amount of gene flow, the genetic architecture and complexity of the studied trait, and the strength and direction of selection. They suggest that future research on parallel clines should consider drift as a null model, to be tested first, prior to attributing a pure role of natural selection to the occurrence of clines along environmental gradients.

For our results on evolutionary trait shifts in *Daphnia*, we do not have strong data to reject the possibility of genetic drift occurring in urban populations. Yet, we have several lines of evidence that make the likelihood of the patterns being explained by neutral dynamics very low. First, we found a cline associated with urbanization in not one but multiple traits. The majority of traits we studied showed a pattern associated with urbanization. This is unlikely to be associated with neutral variation, as it is unlikely that the genetic architecture of all traits would be such that it would foster the occurrence of clines linked to neutral dynamics. Second, the link between the environmental gradient (e.g. urban heat island effect) and the evolutionary trait changes (e.g. heat tolerance) is very direct. Third, most of

the traits that we studied, such as heat tolerance and life-history traits, are likely polygenic in nature and do not show the simple genetic architecture that would foster the formation of clines by neutral dynamics. For example, for the data of a full genome resequencing study of the life-history and behavioral responses of a natural *D. magna* population to changes in fish predation pressure reported upon in the resurrection ecology study by Stoks *et al.* (2016), it was reported that not less than 10% of all genes might have responded in a time span of six years (Chaturvedi *et al.*, unpublished data). This is clearly suggestive of highly polygenic responses underlying the observed life-history trait changes. Finally, this same study and the results by Orsini *et al.* (2017) also suggest that effective population sizes of *Daphnia* populations might often be high.

2 Urban eco-evolutionary dynamics

We have shown pronounced patterns of genetic differentiation along urbanization gradients in the water flea *Daphnia*. It is an important question to what extent such evolution impacts ecological dynamics (Hairston *et al.* 2005; Fussmann *et al.* 2007; Urban *et al.* 2008; Hendry 2017). In the following paragraphs I focus on three aspects of such potential eco-evolutionary feedbacks, and discuss how evolution of higher heat tolerance, smaller body size and faster pace-of-life might influence community composition, responses to global change, and top-down control of algae. For each of these three themes, I discuss results of analyses that were presented in this thesis as well as potential but yet not quantified impacts. For instance, for community composition, we have quantified the impact of intraspecific trait change in body size of *D. magna* in the context of a community-wide shift in body size along the urbanization gradient in zooplankton (Chapter 5; see also Merckx *et al.* 2018). In addition, we carried out a common garden experiment in which we tested predator-prey interaction strengths when both *D. magna* and its predator, larvae of the damselfly *Ischnura elegans*, were isolated from contrasting habitats along the rural-to-urban gradient (Chapter 6). For the feedback of *D. magna* evolution on responses to global change and top-down control of algae, one can make predictions on expected responses based on the evolution of heat tolerance and body size, respectively, but so far these expectations have not yet been tested.

2.1 Eco-evolutionary feedbacks: community context

2.1.1 Zooplankton community composition

As discussed in Chapter 5 we found overall reductions in community-weighted body size across an extended range of 83 communities sampled across the urbanization gradient in Flanders, i.e. sampling the same metacommunity from which we also isolated the *D. magna* clones used in our study. This result is in line with environmental filtering via a thermal gradient and a replacement of large-bodied cladocerans with small-bodied chydorids in the more urban communities (Chown & Duffy, 2015; Gianuca *et al.*, 2018) and was the dominant pattern of community-weighted body size change observed

across 10 phylogenetic groups in Flanders (Merckx *et al.* 2018; Fig. 2). Out of ten studied taxa, the effect size of the zooplankton community response was the largest (Fig. 2, panel J on the left, and lowest plot on the right), indicating a 44% reduction in community-weighted body size along the gradient. As hypothesized in literature (Forster *et al.*, 2012; Horne *et al.*, 2015; Verberk *et al.*, 2016), These results show warming might indeed have more profound effects on body size in aquatic compared to terrestrial communities due to stronger warming- associated oxygen limitation in water.

The overall analysis (including all communities, see Chapter 5) shows clear evidence of zooplankton 'downsizing' in line with the temperature-size rule (TSR; Atkinson, 1194) in response to the higher temperatures linked to the urban heat island effect (Daufresne *et al.*, 2008; Gardner *et al.*, 2011; Edeline, 2013). This supports our Hypothesis 3,as formulated in the Introduction to this thesis ("*Urbanization results in zooplankton community trait change that runs in the same direction as observed evolutionary trait change."*). When we made a distinction between communities dominated by large-bodied species and communities dominated by small-bodied species, however, a different pattern was observed. In communities dominated by large-bodied species, we found an increase in community-weighted mean body size with increasing urbanization. Given that we found the opposite response for evolutionary trait change of the large zooplankton species *D. magna* across the urbanization gradient, the increase in average community body size in zooplankton communities dominated by large-bodied species is somewhat counterintuitive. Any explanation is to some extent speculative, but communities dominated by large-bodied zooplankton, mainly *Daphnia* species, require a sufficient amount of good quality resources (cf. high P-content; Elser & Sterner, 2002), combined with a suitable predator environment (i.e. low fish predation, Kerfoot & Sih 1987);.

For the 12 communities for which we had common garden data of *D. magna*, we could carry out an analysis of the relative importance of different intraspecific and interspecific components to the observed community-weighted body size change of zooplankton in response to urbanization. Key results were that (1) that we observed a large contribution of intra- compared to interspecific trait variation to the observed community-weighted trait change, (2) that the contributions of genetic trait change and phenotypic plasticity in response to temperature were minor, and (3) that the most important component to overall community trait change involved intraspecific trait changes that were not directly related to temperature. This might involve phenotypic plasticity to other environmental factors (e.g. food concentration or quality, presence and activity of predators, …) or ontogenetic shifts. Both the trait change mediated by species sorting (interspecific component) and the net trait change mediated by intraspecific trait variation contributed to the increase in body size with increasing levels of urbanization. We speculate that both are linked to a change in food quality or quantity and/or a change in predatory environment that promotes larger zooplankton because they are stronger competitors (Lampert & Sommer, 2010). In the case of intraspecific trait variation, the observed change in body size might either reflect better food conditions fostering faster growth or a shift in age distribution in

urban compared to rural populations. If urban populations contain less juveniles or on average older individuals than rural populations, then this could explain the observed trait change, given the indeterminate growth of *Daphnia* (Threlkeld, 1987; Martínez-Jerónimo, 2012). It is striking that intraspecific variation in one species can contribute so strongly to community-weighted trait change along environmental gradients. One comment that needs to be made here is that the data set was to some extent biased, as this analysis could only be applied to communities that contained the focal species. In addition, however, it is important to consider that *D. magna* is the largest bodied species in the region, and therefore it has the scope to span a very broad range of body sizes especially if the whole ontogeny is taken into consideration: newborns are approx. 0.7 mm in length, while adults can grow to 5 mm in length if they are well-fed and not eaten by predators. If urban ponds are characterized by high quality food and low predation environments, then they may foster populations dominated by larger individuals than rural populations, and the difference can be large enough to rival differences that one might observe among species.

A second observation is that the evolution for smaller body size that we observed can be viewed as a countergradient response that counteracts the ecological shift to larger body sizes observed in phenotypic trait values. The observed evolutionary trait change thus buffered for the ecological effect, leading to a case of cryptic eco-evolutionary dynamics. It is indeed conceivable that the genetic shift towards smaller body sizes is in part driven by the combined effect of the environmental conditions fostering stronger growth or an age shift distribution towards older and thus larger individuals on the one hand and the selection pressure towards smaller body sizes imposed by the urban heat island effect on the other hand.

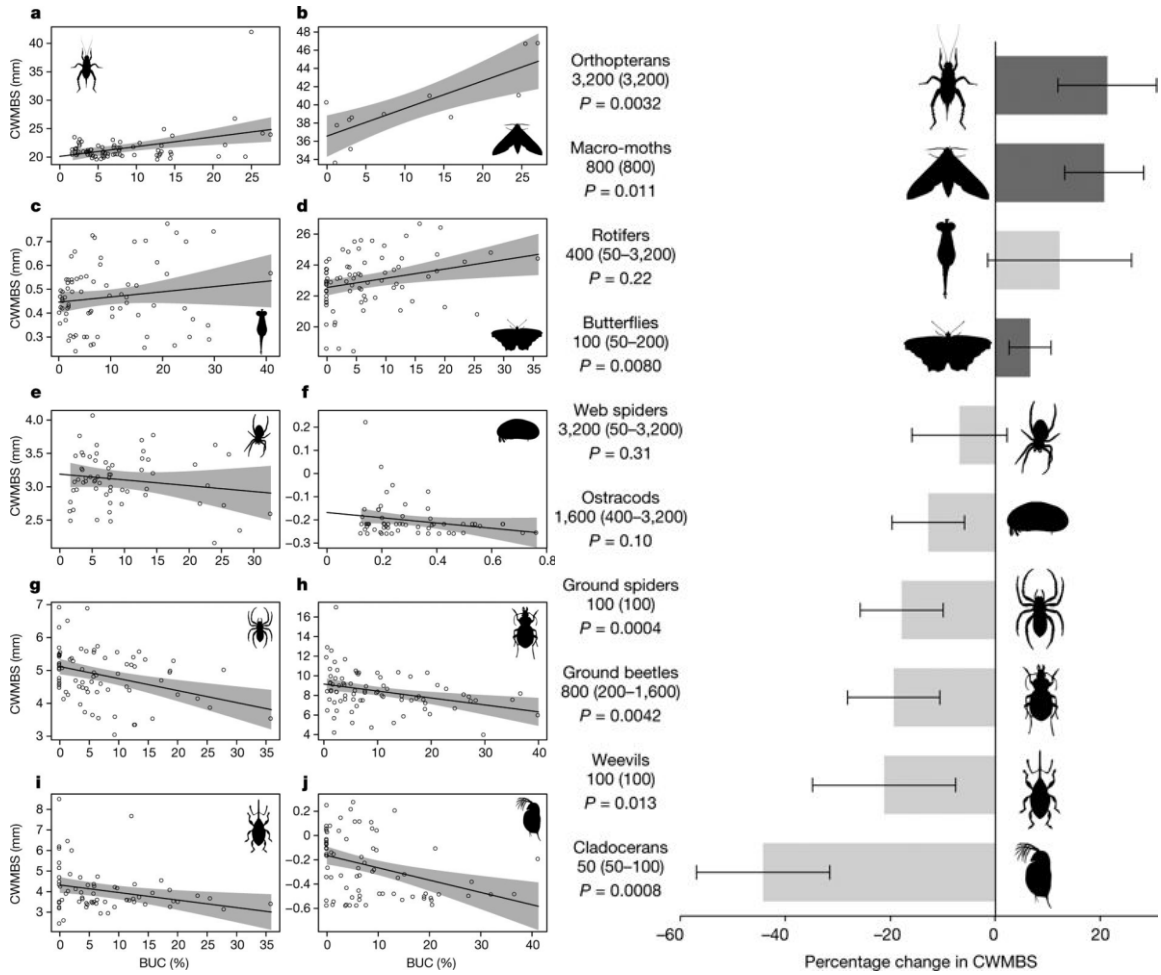

Figure 2. Modelled community-weighted mean body size (CWMBS) based on relative abundance data of species combined with their average body length (left) and extent of the mean percentage change in community-weighted mean body size (right) for 10 taxonomic groups when comparing sites that differed by 25% of built-up area in the study region of Flanders (from Merckx *et al.*, 2018).

2.1.2 Predator-prey interactions under simulated heat waves: urbanization background and cryptic eco-evolutionary dynamics

Empirical evidence on how evolution in multiple interactive species shapes the outcome of ecological interactions such as predator-prey dynamics, host-parasite interactions, and mutualistic associations are more commonly studied in experiments (Becks *et al.*, 2012) or contrasting habitats along large geographic gradients (De Block *et al.*, 2013; see also the review Tüzün & Stoks, 2018b for the possible consequences of the evolution of TPS for biotic interactions under climate change), compared to short distance gradients such as urbanization gradients. In Chapter 6, we set out to test how adaptation to urban settings in predators and prey impact their interaction strength. The results show a significant interaction effect of urban background of both damselflies and *Daphnia* on the rate of predation observed in damselflies. Intriguingly, the interaction effect resulted in a clear case of cryptic eco-evolutionary dynamics (Kinnison, *et al.*, 2015): while urban damselflies captured more prey compared to rural damselflies when offered rural *Daphnia*, this difference disappeared when they were offered

urban *Daphnia*. Urban evolution of *Daphnia* thus counteracted the impact of urban evolution in its predator,

We can only speculate on the mechanisms underlying the observed changes in predator-prey dynamics upon urban evolution in both predator and prey. Although a slower growth and development was observed in urban compared to rural damselflies because of the longer growing season in urban ponds and the associated relaxation of developmental time stress (Tüzün *et al.*, 2017b; and Chapter 6), it might be that local environmental conditions in the city might increase metabolic energy needs in order to cope with an increased exposure to stress. In addition, there is a selection for higher flight endurance of adult damselflies in urban populations (Tüzün *et al.*, 2017c), which similarly might select for an increased foraging activity in larvae (cf. carry-over effects across life stages; Tüzün & Stoks, 2018a; Tüzün *et al.*, 2018) . This increased foraging activity might result in a higher predation rate on rural *Daphnia*. In *Daphnia*, urban evolution of higher heat tolerance, smaller body size, and higher haemoglobin concentrations might reduce the impact of this increased activity of the damselfly larvae. For instance, to the extent that urban water fleas are better adapted to high temperatures, rural water fleas might have suffered more from the simulated heat wave conditions, which rendered them easier to catch. Yet, upon encounter, the dual mechanisms of thermal evolution in both parties decreases predation rates of the urban predator upon exposure to urban water fleas.

The results of our study provide an illustration of the importance of studying the combined effect of (urban) evolution in multiple species and how evolution of one species might in some cases neutralize the effect of evolution in another species. In addition, our results might have interesting consequences in case of range expansions. Urban damselflies have been shown to have a high flight endurance (Tüzün *et al.*, 2017c) and so might be capable of rapid expansion to rural areas if conditions would foster such an expansion (see also 2.3). Urban damselflies would be efficient predators on *Daphnia* in suburban and rural settings, possibly changing trophic dynamics. Whether this might happen and to what extent this would influence the structure of local communities and ecosystems is speculative, and additional work, including modeling and transplant experiments, would be needed to explore this further.

2.2 Eco-evolutionary feedbacks: ecosystem functions

We showed a significant decrease in body length in urban compared to rural *Daphnia* populations (Chapters 2, 3). Body size is a key determinant of grazing efficiency in *Daphnia* (Brooks & Dodson, 1965; Gianuca *et al.*, 2016). A reduction in either size at maturity (Chapter 2 and 3, common garden experiment) or community body size (Chapter 5) might therefore be expected to reduce top-down control on algae. In freshwater zooplankton, body size is both a functional response trait (as it influences the response of species to productivity, pollution, temperature, and fish predation gradients; Brooks &

Dodson, 1965; Moore & Folt, 1993; Gianuca *et al.*, 2016) as well a functional effect trait, as it determines competitive strength and the capacity to graze on algae (Brooks & Dodson, 1965; Sterk *et al.*, 2013; Gianuca *et al.*, 2016). In order to assess to what extent evolution of a smaller body size impacts top-down control of algae and thus ecosystem properties of urban ponds, one needs to not only consider the change in grazing efficiency but also changes in density and competitive strength that result from the evolutionary trait shift in body size. While a smaller body size reduces grazing efficiency and competitive strength, becoming smaller might be key to sustain viable *D. magna* populations in the warm urban habitats. As a result, urban evolution likely led to an increase in top-down control of algae compared to a setting in which evolution would not have been possible, because of the higher densities that can be maintained by the *Daphnia* populations inhabiting urban ponds exposed to the urban heat island effect. Yet, top-down control of algae by evolved urban populations is likely reduced compared to the larger-bodied rural populations. In other words, evolutionary trait change likely achieves a partial but no full buffering of the reduction in top-down control of algae that would result from the urban heat island effect in the absence of evolution. A proper assessment of these effects would, however, only be possible by carrying out transplant experiments.

2.3 Eco-evolutionary feedbacks: global change

The evolutionary trait shifts in heat tolerance and body size that we reported (Chapter 2) are strikingly similar to the trait evolution that has been reported in response to climate change (Geerts *et al.* 2015). In short, the evolution of heat tolerance in response to urbanization reported by us parallels the evolutionary increase in heat tolerance observed in *Daphnia magna* in response to climate change observed over a period of 40 years (resurrection ecology approach; Geerts *et al.*, 2015; see also Yousey *et al.*, 2018 for similar results on *Daphnia pulicaria*). While evolution of heat tolerance in ectotherms has been hypothesized to occur in the future in response to climate change and climate variability, these predictions are mainly based on comparative analyses across altitudinal or latitudinal gradients and extrapolations from warming experiments (Sørensen *et al.*, 2001, 2005; Chown & Gaston, 2008; Deutsch *et al.*, 2008; Somero, 2010; Bozinovic *et al.*, 2011; Sunday *et al.*, 2012; Stoks *et al.*, 2014; Yampolsky *et al.*, 2014; Diamond *et al.*, 2017a; Pörtner *et al.*, 2017; Baudier *et al.*, 2018). Our results on urbanization gradients suggest a similar response over shorter geographic distances (see also Diamond *et al.*, 2018a,b; Kaiser *et al.*, 2016).

The widely studied Bergmann's rule and temperature-size rule (TSR; Atkinson, 1994) are suggested to explain the observed 'downsizing' of communities and populations due to warming associated with climate change (Deutsch *et al.*, 2008; Daufresne *et al.*, 2009; Baudier *et al.*, 2015; but see Teplitsky & Millien, 2013). The evolution of a smaller size at maturity along the urbanization gradient that we report upon in *Daphnia* aligns very well with expectations based on Bergmann's rule

and the temperature-size rule and is supported by similar findings on downsizing along rural-to-urban gradients in a wide range of taxa (Merckx *et al.*, 2018).

Linked to smaller body size, a shift towards a faster pace-of-life has been suggested for ectotherms when reared at higher temperatures or when coming from more warmer, unstable, climates (Zuo *et al.*, 2012; Jaramillo *et al.*, 2017; but see voltinism-associated reverse patterns according to countergradient variation, Conover & Schultz, 1995; Conover *et al.*, 2015; Mikolajewski *et al.*, 2015). Here again, the change towards a faster pace-of-life that we report for *Daphnia* in response to urbanization aligns well with predictions under climate warming. The latter confirms again the similarity between microgeographical and macrogeographical warming gradients. This suggests that studying patterns along urbanization gradients might be valuable for our understanding of future evolutionary and ecological responses to climate warming. Yet, one should interpret these parallels cautiously, as urbanization gradients involve a complex interaction of multiple stressors that might in combination be very different from the stressors associated with climate warming in rural habitats. In addition to differences in environmental selection pressures, there might also be intrinsic differences in evolutionary potential or behavior between urban and rural populations. Amongst others it is important to assess the heritability of thermal tolerance traits and other genetic constraints on evolution (Hoffmann, 2010; De Meester *et al.*, 2017), or differences in behavioral mechanisms that might contribute to the avoidance of exposure to warming (McCauley & Mabry, 2011). For example, in some *Drosophila* species, heat tolerance has been reported to show limited heritability, which can slow down the evolution of this trait (Hoffmann *et al.*, 2003; Hoffmann, 2010; Santos *et al.*, 2012).

3 A brief note on applications for management

The key implications of our work relate to the fields of urban evolution (Alberti, 2015; McDonihue & Lambert, 2015), eco-evolutionary dynamics (Hendry, 2017), and improved predictions of responses to urbanization and climate warming (e.g. Urban *et al.*, 2016). Our observation of extensive levels of genetic differentiation for a broad suit of traits in response to urbanization also reflects, however, that urbanization is a strong selection factor that can strongly impact community characteristics as well as population features. Even if one does not observe changes in population densities in urban compared to rural populations, it might be that this absence of a change in population density hides a change in genetic structure and trait distributions of individuals in the population. Given that such changes might impact ecosystem characteristics and ecosystem services to society, our results provide additional support for proper urban pond management and design.

Important measures would be those that reduce the urban heat island effect and promote conditions that result in clear-water ponds At the scale of urban planning, the establishment of green and blue infrastructure such as parks, ponds and lakes is important to reduce the urban heat island effect

(Völker *et al.*, 2013; Önder & Akay, 2014; Alavipanah *et al.*, 2015). By increasing the amount of ponds and lakes one can also alleviate dispersal limitation, which may foster the establishment of large cladoceran species that are efficient grazers. To improve water quality in the water bodies, they should be surrounded by sufficiently large buffer zones that reduce the direct inflow of water from impervious substrates. Allowing littoral vegetation (e.g. reed or marshland) to develop around the ponds and lakes will further contribute to water quality (Erickson *et al.*, 1994; Suresh & Ravishankar, 2008). In order to reduce the risks of algal or toxic cyanobacteria blooms, one might stimulate the establishment of underwater vegetation or promote successful establishment of large zooplankton. Keeping the ponds or lakes fishless can be key to promote underwater vegetation or top-down control of algae by zooplankton (Scheffer *et al.*, 1993). In lakes with fish, the establishment of a large vegetated littoral zone and the establishment of a fish-free zone might foster clear-water conditions. The morphometry and substrate of the ponds also can be important, as macrophytes will have more difficulties to develop in ponds with vertical or steep walls, and if the ponds have a concrete hull.

4 Some critical reflections and limitations

4.1 Selection of ponds

4.1.1 Sampling design

A key aim of my research was to understand whether or not urbanization drives evolutionary dynamics in organisms inhabiting freshwater ponds, such as *Daphnia magna*. We aimed for elucidating a general pattern that would reflect evolutionary dynamics across cities (Thompson *et al.*, 2016; Winchell *et al.*, 2016). To document patterns of parallel evolution across cities requires an integrated approach, sampling multiple urbanization gradients across multiple cities. Only a few studies, mostly on plants and terrestrial arthropods, successfully applied this sampling strategy (Thompson *et al.*, 2016; Diamond *et al.*, 2018a). This full-fledged sampling strategy was difficult for our study system. First, *D. magna* is not very common in Flanders and it proved difficult to find populations across the whole gradient in multiple transects in multiple cities. Secondly, we aimed at carrying out common garden experiments quantifying multiple traits in multiple clones from each population, and the mere size of the resulting experiments would have made it difficult to study full transects across multiple cities.

Instead of sampling parallel transects across multiple cities, we opted for sampling populations across the urbanization gradient scattered over multiple cities in Flanders, ensuring that ponds situated in areas with low, intermediate and high levels of built-up area were equally spread over the same region. So while we could not sample urbanization gradients across multiple cities, we sampled 13 populations representing a generalized urbanization gradient covering a territory that spans a large part of Flanders. Across Flanders, we thus quantified the impact of urbanization on evolutionary trait change

in replicated urban and rural *Daphnia* populations. The urban populations that we sampled were situated in the major cities of Flanders (i.e. Antwerp, Brussels, Ghent, Leuven and Mechelen). The 13 populations that we used in this study were selected based on three main criteria (fish free ponds; representative levels of built-up area in the intermediate (400 m) to regional (3300 m) perimeter around the pond; no dominance of agricultural land and a minimal amount of biologically valuable land in the low urbanization category; pond needed to be inhabited by *D. magna*; pond needed to be accessible). Our approach is similar to the point sampling strategy reported upon by Winchell *et al.* (2016).

4.1.2 'Metropole Flanders'

Flanders is one of the regions of Europe that is characterized by the highest population density (490 persons/km^2) and is highly urbanized. The region is therefore well-suited to study responses of populations, communities and ecosystems to pronounced urbanization, which was the key aim of the SPEEDY project with which my research was associated (e.g. Merckx *et al.*, 2018). If we want to extrapolate our results to other urbanization areas in the world, however, it might be important to take note of the fact that overall urbanization levels in the whole region are quite high ("metropole Flanders"). For example, the urbanization dynamics in Flanders and most parts of Europe are inherently different from urbanization patterns in North-America and Asia. European cities are often quite old, while North-American cities are in general younger. In contrast, the size of our cities is often only a fraction of the mega-cities found in Asia and North-America (Birch & Wachter, 2011; Taubenböck *et al.*, 2012; Shafizadeh- Moghadam & Helbich, 2015). Additionally cities in Europe are often spatially structured in a different way than in other areas of the world (urban agglomerations; see also Introduction, and Poelmans, 2010, PhD dissertation). In Flanders, cities contain a distinct urban core (i.e. 'city center') with a peri-urban area, and those polycentric urban areas are linked through transportation and ribbon development. Cities in the United States, Canada and Australia are often more open, geographically larger, but dispersed formations of urban area, with large suburban zones and industrial intersections, but often also with large patches of parkland or natural area. So urbanization gradients may differ according to the region in the world studied.

In addition, given that Flanders has been densely populated since the onset of the Industrial Revolution, the whole landscape has been strongly modified by human activities and the small remaining natural areas cannot be considered truly pristine. As a result, our rural ponds may be less pristine than rural ponds in many other areas of the world. This is not an essential problem for our study, as we do report strong gradients in temperature between urban and rural ponds and strong genetic differentiation between urban and rural populations in multiple traits. In other areas, some of the gradients might be even more pronounced than the ones we report upon in Flanders.

4.1.3 Characterization of the urbanization gradient

We characterized the urbanization gradient by percentage built-up area. In a parallel study (Engelen, 2017, PhD dissertation), the environmental characteristics of a total of 83 urban and rural ponds were quantified during a survey. Urbanization had a surprisingly small effect on environmental features of the ponds, likely because both urban and rural ponds can vary strongly in their features. One important limitation of this dataset is, however, that we only obtained a snapshot survey. It might be that we would be able to characterize the ponds much better by a regular monitoring of a set of urban and rural ponds. One interesting idea would be to integrate such a monitoring effort in a Citizen Science project.

4.2 The choice of traits studied and the experimental design

Urban evolutionary studies rarely include more than one or two focal traits. Yet, a multi-trait approach is crucial for an integrated understanding of the impacts of urbanization on life-history, behavior, physiology, immunology, and stress tolerance. In the present study we characterized urban evolution in not less than 17 traits using targeted statistical approaches to achieve an integrated analysis (Collyer & Adams, 2007; Adams & Collyer, 2009; Rosseel, 2012; Lefcheck, 2016; Stoks *et al.*, 2016). Next to measuring CT_{MAX}, we assessed other traits such as life history (size at maturity, development time, fecundity; Chapter 3, 5) and physiology (energy reserves, oxidative stress responses; Chapter 4) at two environmentally relevant temperatures (20 and 24 °C), mimicking the observed urban heat island effect during spring and summer across the studied urbanization gradient (see Chapter 1).

4.2.1 Broadening the temperature range

Carrying out a common garden approach at two temperatures enabled us to partition evolutionary and plasticity responses, and test for the evolution of plasticity (see also Diamond *et al.*, 2018a). However, a valuable extension of our approach would be to study life-history and physiological traits across a gradient in rearing temperatures rather than just two. For example, we could have analyzed trait variation across multiple temperatures spanning 16 °C to 30 °C (see McLean *et al.*, 2005; Tüzün *et al.*, 2017b; Diamond *et al.*, 2018a). Such an approach would have enabled us to asses thermal performance curves (TPCs) for multiple functional traits (Conover & Shultz, 1995; Conover *et al.*, 2009). Temperature performance curves can be directly linked to fitness and performance traits and provide a more clear perspective on whether observed responses represent cogradient or countergradient responses (Fig. 3). In addition, an assessment of temperature performance curves would enable us to make clear-cut inference on how good CT_{MAX} values reflect horizontal shifts in TPCs, which would support the use of CT_{MAX} as a good proxy for overall fitness properties (Fig. 3). Quantifying thermal performance curves would also enable one to assess whether differences in thermal buffering windows (i.e. the difference between CT_{MAX} and environmentally experienced temperatures) have consequences for functionality at lower temperatures in both urban and rural populations. While CT_{MAX} and CT_{MIN}

are often criticized for not being good performance traits or environmentally relevant response traits, they do constitute the endpoints of the temperature performance curve and thus represent a thermal window that organisms can tolerate. Growth and fecundity are crucial for fitness at intermediate temperatures, but tolerating extremes is also essential, given that, as temperatures rise (from CT_{MIN}) or decrease (from CT_{MAX}), towards T_{OPT}, growth and reproduction can restart and population survival is guaranteed (Overgaard *et al.*, 2014). In addition, CT_{MAX} measurements allow to calculate thermal buffers (i.e. CT_{MAX} minus the most extreme summer temperature observed), which are expected to decrease under climate change (Buckley & Huey, 2012; Kinsgolver *et al.*, 2013; Sunday *et al.*, 2014; Deutsch *et al.*, unpublished data), and can be initial proxies to estimate vulnerability to increased warming. A third benefit of assessing thermal performance curves is that measuring the response at high temperatures would enable us to specifically check the plasticity of urban populations as they are exposed to increasing temperatures. Currently it is not sure where the limits of acclimation lie in *Daphnia* (e.g. see Kellerman & Sgrò, 2018).

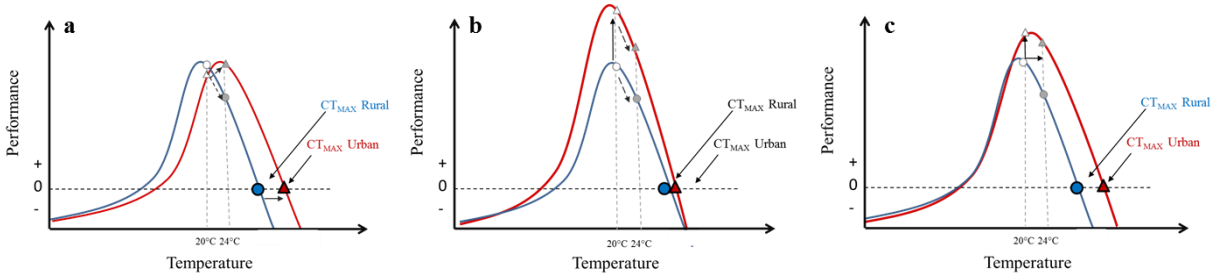

Figure 3. Example of possible thermal performance curves (Conover & Shultz, 1995; Conover *et al.*, 2009) showing a horizontal (a), vertical (b), and mixed vertical and horizontal (c) genetic shift (black arrows), including expected shifts in Critical Thermal Maxima and performance in the 20-24 °C thermal window (grey open and filled symbols respectively, connected via dashed reaction norms). The results of our study largely support scenario c, but including more rearing temperatures would enable us to reconstruct the entire thermal performance curve and validate whether or not the evolutionary response in urban populations observed for each of the studied traits aligns with the hypothesis of cogradient variation.

4.2.2 Broadening the range of traits

Although our multi-trait approach covered a broad suit of traits relevant in the framework of urban evolution, a set of traits remain unstudied (Fig. 4). For example, heat shock proteins (HSP; Feder & Hofmann, 1999). Heat shock proteins are chaperone proteins that protect other biomolecules from denaturation. Their concentration has been shown to increase in response to numerous environmental stressors such as predation, metal contamination, and warming (Feder & Hofman, 1999), and several studies have shown that *Daphnia* populations can evolve in terms of their constitutive level of heat shock proteins (Pauwels *et al.*, 2007; Zhang *et al.*, 2016). Quantifying concentrations of heat shock proteins in urban and rural *Daphnia* populations would potentially contribute to clarifying our results on stress physiology and the POLS presented in Chapter 4. Thermal physiology has been suggested to play a major role in shaping plastic and evolutionary pace-of-life changes in ectotherms in such a

manner that the slow-to-fast axis can be aligned to a cold-to-warm axis (Goulet *et al.*, 2017). For example, the reduced activity of SOD and GST, yet similar levels of oxidative damage in urban compared to rural populations (Chapter 4), could be the result of a genetic difference in basal heat shock protein content between urban and rural populations. Given that heat shock protein production is initiated under a variety of anthropogenic and natural stressors, urban environments that are affected by a number of different abiotic and biotic disturbance including the urban heat island effect might have selected for higher constitutive heat shock protein production. In this context, another set of potentially highly relevant, but so far unstudied traits, are those linked to mitochondrial energy metabolism, for example the electron transport chain activity (ETS; De Coen & Janssen, 2003; Dinh *et al.*, 2016) and metabolic activity (e.g. respiration rates at different temperatures; Simčič & Brancelj, 1997; Lamkemeyer *et al.*, 2003; Paul *et al.*, 2004). The cold-hot axis in POL studies has been only explored in a few studies (Goulet *et al.*, 2017; Chung *et al.*, 2018; Goulet *et al.*, 2018; Michelangeli *et al.*, 2018).

Most studies on ecological and evolutionary pace-of-life responses to urbanization focus on an integration of behavioral, life history, and physiological traits (e.g. Charmantier *et al.*, 2017; Tüzün *et al.*, 2017a; Sprau *et al.*, 2017; Sepp *et al.*, 2017). While I initially considered this less relevant for *Daphnia*, assessing behavioral traits such as phototactic behavior, which is associated with diel vertical migration (DVM) might be relevant in the context of urban evolution. *Daphnia* migrate during the day to deeper layers of the water column to avoid visual predators such as fish or to avoid damage from UV light (Brooks & Dodson, 1965; Stich & Lampert, 1984; Lampert, 1989), while they move up during the night to feed and to maximize reproductive and developmental rates through nighttime exposure to warmer near-surface water (Dodson *et al.*, 1997b). Given that diel vertical migration has a strong cost (avoidance of warm and food rich water layers during the day), populations differ in the extent to which they perform diel vertical migration dependent on the need to avoid surface water layers during the day to avoid UV light or predators. Even species inhabiting shallow lakes can show pronounced patterns of diel vertical migration, and it has been shown that *Daphnia magna* populations can differ strongly in phototactic behavior and daytime position (De Meester 1993a, 1993b, 1996). Diel vertical migration has been shown to evolve in populations exposed to fish predation (Cousyn *et al.*, 2001; Stoks *et al.*, 2016). It is conceivable that diel vertical migration might evolve in response to warming, as deeper water layers are often somewhat colder than surface layers. While the scope for migration to reach less warm water layers is largest in deeper lakes, in such lakes there is a conflict with food availability, as phytoplankton growth is limited to the photic zone. Shallow lakes are generally well mixed so that the scope for finding cooler water closer to the bottom is more limited, but residing in water with 0.5 or 1 °C lower temperature might make a difference during periods of very warm weather. Interestingly, a life-history study showed that clones that remain in more surficial water layers show a smaller body size and faster development time than clones that migrate, in line with the pace-of-life axis and with the features of the urban populations that we studied in this thesis. In the case of diel vertical migration,

these trait shifts have been interpreted as a response to an increased predation risk by visual predators (fish, selecting for both small body sizes and high reproduction rates). The shift to a small body size and faster pace-of-life would, however, also enable the animals to deal with higher temperatures.

In addition to a further exploration of physiological traits underpinning adaptation to urbanization, it would be interesting to test whether there is genetic differentiation in the frequency of sexual reproduction (i.e. the formation of ephippia) between urban and rural populations. *D. magna* produces sexual eggs when conditions deteriorate, mostly before winter, but also when resources become limiting due to overcrowding, or in response to the presence of predators (Carvalho & Hughes, 1983; Kleiven *et al.*, 1992). Given that urban habitats are likely more exposed to mechanical and chemical disturbances linked to management and a high amount of human activity in their immediate surroundings, urban habitats might select for a higher responsiveness to environmental cues inducing the production of males and sexual, dormant eggs.

Finally, we observed that the shift to a faster pace-of-life in urban *D. magna* populations results in a higher population growth rate at the two temperatures studied (Chapter 2-4). To the extent that population growth rate under non-limiting conditions (i.e. the conditions at which we quantified life-history traits in our common garden experiment) is a good measure of fitness in the field, this results in a paradox: why do not all populations show this shift to a faster pace-of-life? This apparent paradox likely reflects that our laboratory conditions fail to grasp key parts of the complexity of the environment in which the *Daphnia* populations develop, so that we were not able failed to document the whole set of trade-offs with other responses that shapes the slower pace-of-life strategy in rural populations. The observed fast-life strategy and investment in heat tolerance likely comes at a cost. We did not find such costs or trade-offs between heat tolerance, life history traits and physiological traits such as oxidative stress responses. There are several possibilities for such costs, which we did not yet, however, explore. First, the amount of energy reserves invested per juvenile might be lower in urban compared to rural populations, which might reduce their survival under low-food conditions. Alternatively, adults of urban compared to rural populations might show a reduced longevity (i.e. reduced number of molting and reproduction events) or produce fewer juveniles in later clutches (Patrick & Weimerskirch, 2015; Lemaître & Gaillard, 2017). The latter mechanism has recently been suggested to solve the pace-of-life puzzle in which fast living organisms do not die fast, but rather reproduce poor quality or fewer offspring later in life. In our case, second generation life table experiments were not performed, and mother individuals were observed until the release of third clutch, which does not allow to assess these suggested costs. In a broader context, it might be that the faster pace-of-life is traded off with investment in energy in mechanisms to cope with biotic interactions. For instance, if rural populations would be exposed to higher abundances or a more diverse set of parasites, then rural populations might be selected to invest more in immune responses compared to urban populations. Alternatively, gape limited invertebrate predators such as the phantom midge larva *Chaoborus* is known to select for a life history

characterized by a larger size at maturity coupled with the production of less but larger juveniles and a slower development time (Lüning & Stibor, 1994; Tollrian, 1995; Tollrian & Harvell, 1998). As such, the slower pace-of-life that we observe in rural populations would be a suitable strategy in habitats with high densities of phantom midge larvae. Finally, if competition for food would on average be higher in rural compared to urban populations, this would also lead to a selection for larger individuals, because they are shown to have a higher starvation resistance (Tessier *et al.*, 1983) The cost of a faster pace-of-life in this context would be that the resulting animals are, either because of their small size or because they do not have enough energy to invest in defense mechanisms, more vulnerable to parasites, and predators or are inferior competitors. In our study, we did not quantify parasite abundances or richness, nor did we have adequate assessments of densities of invertebrate predators such as *Chaoborus*, food quality and the competitive environment in rural compared to urban ponds. As a result, we cannot properly quantify these potential trade-offs.

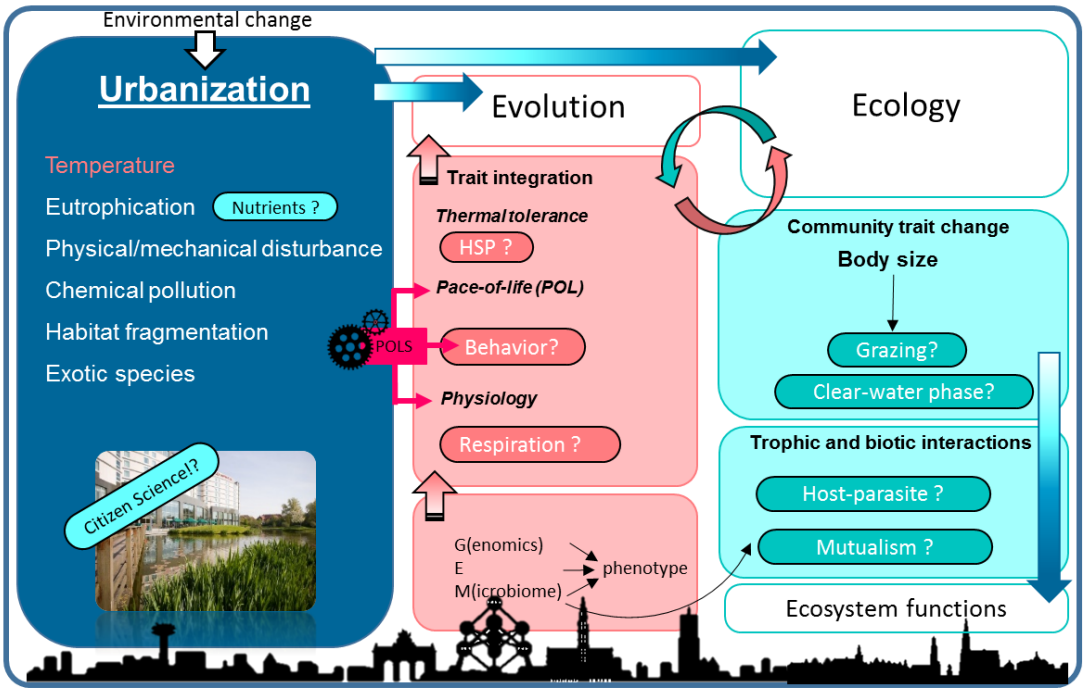

Figure 4. Schematic overview of some limitations of our study and future perspectives in urban evolutionary biology and eco-evolutionary dynamics research in our study system. HSP = Heat Shock Proteins.

5 Future perspectives

In the list of limitations discussed in the previous paragraphs, I suggested a number of additional investigations that could significantly improve our insight into the system. These involved the study of additional traits, a better characterization of the urban gradients, and an analysis of how differences in the biotic environment between urban and rural ponds might structure trade-offs along the urbanization gradient (see also Fig. 4). In the following I want to present a number of research lines that I consider key to obtain better insight in the broad field of urban evolution and urban eco-evolutionary dynamics,

and I translate these into a research agenda for our model system, being water fleas inhabiting ponds along the urbanization gradient (Fig. 4). These research lines include investment in assessing the mechanisms underlying evolutionary responses to urbanization. We here propose applying a broader multi-trait and multi-species approach and extending current urban genomics (5.1). Additionally, the exploration of how microbiomes shape Genotype by Environment interactions and how these Genotype x Environment x Microbiome (G x E x M; Fig. 4) impact trait responses to urbanization might yield important insights into how populations respond to urbanization. Furthermore, there is a need to assess local and regional scale ecological consequences of genetic differentiation between urban and rural populations (5.2). These include eco-evolutionary dynamics in local urban communities also considering host-parasite, predator-prey, and mutualistic interactions, local scale ecosystem functioning, and the consequences of urban evolution in the light of future climate change and range expansions. I discuss these perspectives in the light of the findings of my PhD thesis, with a focus on urban ponds and *Daphnia magna* (see Fig. 4).

5.1 Elucidating mechanisms underpinning urban evolution

5.1.1 Multi-trait approach and pace-of-life syndromes

Future studies in urban evolutionary ecology will benefit from a further development of a multi-trait approach. While initially urban evolution studies mainly focused on one or two traits, more recent studies are increasingly integrating more focal traits (e.g. Jung & Threlfall, 2018; this thesis). The Pace-of-life Syndrome (POLS) framework offers a promising framework to study and interpret trait covariation patterns across gradients, among individuals within populations, among populations, and among species. Comparative analyses of multi-trait responses might shed more light on taxon-specific responses to urbanization, the functional traits involved in evolutionary responses, evolutionary trade-offs and limitations to evolutionary change, and how these are linked to features of the taxon (e.g. body size, dispersal capacity, production of dormant stages, generation time).

5.1.2 Genomics

While some recent efforts have been made to study parallel evolution in the context of urbanization (Thompson *et al.*, 2016; Winchell *et al.*, 2016; Diamond *et al.*, 2018a), empirical evidence for parallel evolution is still limited. Genomic approaches can offer an important possibility to increase the scale of data acquisition and enhance the interpretation of evolutionary clines. Quantitative genetic and genomics analyses reinforce each other. Population genomics studies can in most cases not relate genomic signals to actual phenotypic fitness-related endpoints of evolution. Exceptions are the rare cases where specific genomic markers have been shown to have a large effect of phenotypic variation for a specific trait (Jones *et al.*, 2012; Lamichaney *et al.*, 2015, 2016) Conversely, assessing the genomic

underpinning of observed evolutionary clines in traits gives insight in the relative importance of selection versus neutral evolutionary processes that might be involved (Santangelo *et al.*, 2018).

For *Daphnia*, full genome sequencing of the populations in the common garden studies of this thesis would enable to obtain insight into the genomic basis of the observed evolutionary divergence between urban and rural populations. Genome sequencing, based on restriction site associated DNA sequencing (Colbourne *et al.*, 2004, 2005, 2011), is followed by outlier analysis to identify variable sites that are more strongly differentiated between urban and rural populations than background noise (reflecting neutral evolution). Once identified, genes linked to outlier loci can be annotated based on efforts of the the *Daphnia* Genome project (*Daphnia* Genomics Consortium, see wFleabase.com; Colbourne *et al.*, 2005). Such an approach allows to validate which genomic regions or genes underpin trait shifts along the urbanization gradient (Horowitz, 2007; Simon *et al.*, 2011)**.** In addition, the genome-based approach has the power to detect a much broader set of responses than can be assessed by traditional common garden experiments (e.g. genes linked to specific pathways or physiological and biochemical traits that are difficult to quantify on individual *Daphnia*). Importantly, genomic analyses across multiple contrasting urban and rural population pairs provide a direct test for the degree of parallel evolution, as it can detect whether outlier loci are shared among population pairs.

A key advantage of a genome-based approach is that it can be applied on a much larger scale in terms of number of populations than the labour-intensive common garden approaches. Once we can identify associations between specific genomic signals and trait variation by screening genomic variation of the clones used in common garden experiments, we might be able to interpret genomic data of a larger set of populations along urbanization gradients in other regions of Europe and beyond. *D. magna* has a large species range distribution covering most of the Holarctic (but relatively rare in North America) and Africa (mostly reported from northern Africa including the horn of Africa, and in South Africa: Benzie 2005; De Gelas & De Meester, 2005; Yampolsky *et al.*, 2014). Such a study would involve sampling populations along urbanization gradients associated with different cities and screen for parallel signals of evolution at the genome level. Such an approach would also allow quantifying latitude x urbanization interactions. Given the latitudinal temperature cline, it would be interesting to explore whether genomic signals of evolutionary responses to urbanization are similar across latitudes, and test the idea that responses to urbanization at more northern localities might show similarities to latitudinal clines.

In addition to analyses of genomic variation in nuclear DNA, assessing changes in mitochondrial genes might be highly relevant in the context of urbanization. Mitochondria are not only important contributors to bioenergetics (production ATP), but also have important functions in detoxification, immunology, and stress adaptation via cellular H_2O_2 signaling and ETS fluxes (Iftikar, 2014, 2015; Roubicek & Souza-Pinto, 2017; Leo *et al.*, 2017; Chung *et al.*, 2018; Sokolova, 2018).

Stress-tolerant species and populations are often characterized by , specific mitochondrial haplotype identities (e.g. the 'mitochondrial climatic adaptation hypothesis', Camus *et al.*, 2017 and references therein) or changed levels of mtDNA copy number variation (Dixon *et al.*, 2015; Tsai *et al.*, 2016; Camus *et al.*, 2017; Sokolova, 2018). Mitochondrial efficiency (oxidative phosphorylation, proton leak, and ATP production) is, for example, higher in Atlantic cod compared to polar cod upon warming, suggesting that the latter has lower acclimation potential to warming (Leo *et al.*, 2017). In another example, tidal mollusks are more heat- and hypoxia-tolerant via an upregulation of ETS activity under hypoxia and a fast ETS increase under reoxygenation, while maintaining ATP production (Cherkasov *et al.*, 2006; Ivanina & Sokolova, 2013, 2016; Meyer *et al.*, 2013; Ivanina *et al.*, 2016; Sokolova, 2018). Such results indicate an important role of mitochondrial functions in thermal adaptation.

5.1.3 The role of the microbiome in urban adaptation

It has been hypothesized that gut microbiome-mediated responses contribute to phenotypic plasticity, but potentially do so in an interaction with the host genotype (Macke *et al.*, 2016). This idea highlights the need to revisit the conventional Genotype x Environment (G x E) interactions in evolutionary ecology and incorporate a (gut) microbiome-driven component (M). There is thus a need to quantify G x E x M interactions (Fig. 4). Urbanization might strongly impact the diversity and community composition of microbial communities in terrestrial and aquatic ecosystems (Marzinelli *et al.*, 2018). In addition, warming has been shown to reduce the strength of host-parasite and host-symbiont interactions (Blanford *et al.*, 2002; Hance *et al.*, 2007; Furlong & Zalucki, 2017; Corbin *et al.*, 2017). A recent study in Flanders *et al.* reported reductions in species diversity of microbiota and changes in community structure and functional characteristics in the microbiota of urban compared to rural sparrow populations (Teyssier *et al.*, 2018). Intriguingly, the strength of the relationships were dependent on the season and differed depending on the spatial scale at which urbanization was quantified (Teyssier *et al.*, 2018). Interestingly, the species composition of the gut microbiome of urban birds suggests that it is better equipped for detoxifying potentially harmful xenobiotics compared to the gut microbiome of rural birds. Mukherjee *et al.* (in preparation) similarly found enrichment of genes associated with pathways that are linked to the decomposition of xenobiotics in urban compared to rural bacterioplankton communities. Macke *et al.* (2017) reported that the ability of *Daphnia* to cope with toxic cyanobacteria depends on the gut microbiome, but also that the composition of the gut microbiome is in part linked to the *Daphnia* genotype. These results, in combination, indicate a potentially important role of microbiome x genotype and microbiome x genotype x environment interactions in the response of *Daphnia* to environmental challenges, including urbanization. We propose to study genotype- and microbiome-driven phenotypic shifts in *Daphnia* and assess to what extent they contribute differentially to phenotypic trait change, community composition, and possibly top-down control of algae in urban compared to rural populations (Fig. 4). This can be achieved through gut microbiome transplant

experiments (Macke *et al.*, 2017) combined with transplant experiments of the resulting *Daphnia* lineages in urban and rural ponds.

5.2 Eco-evolutionary feedbacks

5.2.1 Eco-evolutionary feedbacks of urban evolution on community features

Eco-evolutionary dynamics research has received considerable attention over the past decades (Hairston *et al.*, 2005; Urban *et al.*, 2008; Pelletier *et al.*, 2009; Schoener, 2011; Hendry, 2017). While this research has been extremely valuable to better understand the relative contributions of evolution and ecology to observed changes in population, community, and ecosystem features in response to environmental change, much of the research is based on theoretical models or proof-of-principle laboratory experiments in highly simplified systems, and thus often lacks a realistic level of natural complexity (Yoshida *et al.*, 2003; Fussmann *et al.*, 2007; Johnson *et al.*, 2009; Moya-Laraño *et al.* 2012; Bocedi *et al.*, 2014; Pantel *et al.* 2015; Patel & Schreiber, 2015; Vanoverbeke *et al.*, 2016; Alberti *et al.*, 2017b; Hendry, 2017; Patel *et al.*, 2018). Studies that do include interacting species in natural communities often only include evolution in one species (Pantel *et al.* 2015), and thus still are critical steps away from addressing eco-evolutionary feedbacks in a truly multi-species community context (De Meester *et al.*, in revision). Future work should focus on the impact of urban evolution in multiple species, preferentially all common or dominant species in local communities, on community dynamics and ecosystem functions. Given the fast rates at which evolutionary change can take place (Hendry 2017) and the growing number of reports on evolutionary responses to urban settings (Johnson & Munshi-South, 2017), urban evolution might be a convenient model system to further develop research quantifying eco-evolutionary dynamics.

I did assess how evolutionary trait change of our focal species *D. magna* influences community-weighted trait values in a zooplankton metacommunity sampled along urbanization gradients (Chapter 5), and, in addition, to what extent urban evolution in both predator and prey impact predator-prey interactions (Chapter 6). However, our insight is still limited. Our analysis of the impact of urban evolution on community trait values merely quantified the contribution of evolution on trait values for the existing metacommunity in a single survey. Such a survey-based approach does not allow one to quantify whether urban evolution influenced community composition itself. For reaching more insight into this, one needs to do transplant experiments. Our analysis of predator-prey interactions yielded intriguing insight into a possible case of cryptic evolution, but is based on standardized laboratory experiments and thus does not take environmental differences between urban and rural pond environments into consideration. In addition, we do not have data yet on the potential impact of urban evolution on host-parasite and mutualistic interactions. Empirical studies have provided clear evidence for host-parasite co-evolution in *Daphnia* (e.g. Decaestecker *et al.*, 2007, 2013) and there are strong

indications for genotype x gut microbiome interactions (Macke *et al.*, 2017). Both might offer unique model systems to quantify the feedback of urban evolution on interaction strengths.

5.2.2 Eco-evolutionary feedbacks of urban evolution on ecosystem features

Research on how urban evolution feeds back to codetermine ecosystem functions is still absent. Relevant ecosystem functions that might be impacted by urban evolution might include pest-control, nutrient (re)cycling (e.g. see soil property studies: Oktaba *et al.*, 2014; Herrmann *et al.*, 2018), top-down control of algal blooms, and disease spreading (Bezirtzoglou *et al.*, 20011; Neiderud, 2015).

In the framework of our study system, one of the most obvious ecosystem features that is relevant for pond ecology, as well as pond management and the value of ponds to society, is top-down control of algae by zooplankton, contributing to the stabilization of the clear-water state (Scheffer *et al.*, 1993; Jeppesen *et al.*, 2000) and the prevention of (toxic) algal blooms (Kosten et al 2012; Huisman *et al.*, 2018). So far, the impact of urban evolution on top-down control has not been quantified, even though evolution towards a smaller body size can be expected to impact grazing efficiency. I here propose targeted transplant experiments, where reciprocal transplants are carried out between populations from urban and rural habitats, as a powerful way to quantify the impact of urban evolution on ecosystem functions. Transplant experiments allow to also quantify population dynamics and integrate the impact of both altered population dynamics and evolutionary differences in functional effect traits, such as grazing efficiency. The latter is relatively easy to quantify via in situ short-term grazing experiments with a standardized number of *Daphnia*. This would result in a clear assessment of ecosystem functioning throughout a longer period of time and the influence of urban eco-evo dynamics hereto. In addition, given that multiple species in the community likely evolve simultaneously upon a change in urbanization level, it might yield important added insight to include treatments in which the co-evolved community is transplanted along with the focal species. Finally, transplant experiments in enclosures in source and target habitats allow quantification of population, community, and ecosystem features simultaneously. Although urban *Daphnia* evolved a smaller body size and this could impact their top-down control on algae (Gianuca *et al.*, 2016), one expectation we have is that urban *Daphnia* might reach higher population densities or reach carrying capacity faster compared to rural *Daphnia*. Hence, ecosystem functions might not be impaired. A two-month laboratory experiment in which we monitored algae concentrations in small aquaria inoculated with urban and rural *Daphnia* populations suggests such mechanisms to occur (Brans *et al.*, unpublished data). While urban *Daphnia* were significantly smaller than rural ones in this experiment, *Daphnia* cohorts, which were reared at three different temperatures (20 – 24 – 28 °C) did not show differences in the amount of top-down control of algae . *Daphnia* cohorts containing a mix of urban genotypes reached higher densities than those that had a mix of rural *Daphnia*, thus explaining the absence of any grazing difference. Testing ecosystem functions in a natural set-up, integrating interactions with other guild members as well other

biotic interactions (cf. diverse bacterial and algae communities, the invertebrate predator community) is needed to make more relevant assessments of the impact of urban evolution on ecosystem features.

5.2.3 Eco-evolutionary feedbacks of urban evolution on responses to climate warming

As mentioned before, the evolutionary trait changes for thermal tolerance and body size that we observed in the context of urbanization are very similar to evolutionary changes in response to climate change (Geerts *et al.* 2015). It is therefore an important question to what extent urban evolution might render urban populations pre-adapted to climate change. To the extent that urban populations are indeed pre-adapted to climate change, they might extend into rural areas upon climate change and replace local populations, profoundly changing landscape genetic structure. The results of our research indeed are suggestive of competitive advantages of urban compared to rural populations upon range expansions under warming scenarios: they show increased heat tolerance (Chapter 2), faster pace-of-life and population growth rate (Chapter 3), and a higher amount of energy reserves (Chapter 4). The 4 °C temperature increase by 2100 predicted by the IPCC RCP8.5 scenario (global-mean surface air temperature change for 2081–2100 is predicted to range between 2.6 °C– 4.8°C under RCP8.5; UN, 2014), matches the approximate temperature difference that we observed between city ponds and rural ponds in Flanders during the summer season (Chapter 1). We hypothesize that adaptation to urbanization might impact regional population assembly dynamics via pre-adaptation and evolution-mediated priority effects. I suggest to test this hypothesis via the individual-based modelling (IBM) platform RangeShifter (Bocedi *et al.*, 2014). RangeShifter was developed to predict species range changes under realistic climate change scenarios, via expansion simulations in spatially explicit landscapes using land-cover maps, habitat suitability maps, or artificial maps. The model integrates complex population and dispersal dynamics. The platform will be expanded so that it can explicitly take plastic and evolutionary trait changes into account. As an IBM, it accounts for demographic and dispersal stochasticity, and additionally allows inter-individual (and thus also clonal/genetic and plastic) variability in life-history traits. The software handles real maps and GIS information, so that it is possible to simulate responses to climate change in realistic landscapes with explicit inclusion of, for example, urbanization gradients. To implement urban evolution, we can incorporate data obtained from a common garden experiment with 20 urban and 20 rural clones of *Daphnia magna* that we reared at different temperatures in the lab (17-20-23-26-29 °C), and scored for different life-history traits (age at maturity, size at maturity, fecundity, timing of clutch release, somatic growth, and intrinsic population growth rate; Brans *et al.*, unpublished data).

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Summary/Samenvatting

Summary

Urbanization causes substantial environmental alterations, inducing rapid evolution. A key feature of urban areas is their higher temperature ('urban heat islands'), yet evidence on the presence of this phenomenon and possibly adaptive evolution to such anthropogenic warming in small freshwater habitats is extremely limited.

We monitored water temperature in urban and rural ponds in the region of Flanders (Belgium, yearlong study) and found urban ponds to be significantly warmer (minimum, maximum, mean temperatures) and experience longer estimated growing seasons. We conducted large-scale common garden experiments to test for genetic differentiation in a suit of traits, including heat tolerance (CT_{MAX}), life history traits (age at maturity, size at maturity, development time, intrinsic population growth rate, somatic growth), stress physiology (concentration of fat, proteins, sugars, antioxidative defense via enzymatic activity, oxidative damage, haemoglobin concentration) between replicated urban and rural populations of the water flea *Daphnia magna* (13 populations in total).

Compared to rural animals, urban *Daphnia* evolved a higher heat tolerance (up to 2 °C) and a faster life style. Multivariate phenotypic trajectory analyses indicate a genetic life history differentiation along the slow-to-fast axis of the 'pace-of-life continuum' coinciding with early maturation times, smaller size at maturity (in line with the temperature-size rule), higher fecundity and higher intrinsic population growth rates. Furthermore, stress physiological responses show that urban animals have higher concentrations of fat, protein, and sugars, suggesting the evolution of higher levels of energy reserves. In addition, while urban water fleas show a significantly lower activity of antioxidant enzymes, lipid peroxidation (oxidative damage) did not differ between the two genotype sets, suggesting that *Daphnia* evolved a more efficient antioxidant defense machinery in response to urbanization. Applying a structural equation modelling approach on this set of life history, morphological, and stress physiological traits indicated that all traits were significantly structured by an underlying Pace-of-life Syndrome (POLS) in the urban, but not the rural populations. We hypothesize that the combined mix of stressors (temperature, pollution, pond management) and overall different selection pressures experienced by animals in city ponds compared to rural ponds might shift energy allocation to align stress physiological coping-mechanisms to life-history evolution in the direction of fast life, evoking a strong POLS. In rural areas energy might be allocated to, for example, predator defense or immunology. Combined, these results indicate that urbanization drives the evolution of increased heat tolerance, pace-of-life, stress resistance, and pace-of-life syndromes.

Analysis of zooplankton community data, integrating relative abundances and species-specific body size measurements along an urbanization gradient comprising 83 ponds indicated a similar reduction in average community body size, thus indicating evolutionary (i.e. at the population level) and ecological (i.e. at the community level) trait shifts in response to urbanization are remarkably

similar and indicate ‘downsizing’, most likely as a response to increased temperatures in these urban ponds. Yet, evolutionary and ecological responses are clearly shaped by other environmental disturbances such as eutrophication, pollution, or changes in predation pressure.

To test for a possible effect of urbanization-driven evolution in multiple species on ecological dynamics (i.e. ‘eco-evolutionary feedbacks’) we performed a large common garden rearing experiment with both urban and rural water fleas (*Daphnia magna*) and damselfly larvae (*Ischnura elegans*), a common predator of the water flea. Predators and prey were exposed to each other in mild to intense simulated heatwaves according to a reciprocal crossing of their evolutionary (urban) background (i.e. urban x urban, urban x rural, rural x urban, rural x rural). Results indicate an (asymmetrical) ‘mismatch’ effect; while urban predators show a higher predation rate compared to rural predators when exposed to rural *Daphnia*, this predation rate advantage disappears when exposed to urban water fleas, indicating the urban, and thus evolutionary background, of both interactors shapes the result of their interaction (i.e. predation rate). These results are an important example of cryptic eco-evolutionary dynamics, showing that evolution in one species buffers for the ecological consequences of evolution in the other species). We hypothesize the signals of thermal adaptation earlier observed in both predator and prey are the main drivers of these cryptic eco-evolutionary dynamics and the observed mismatch effect. Detecting such cryptic eco-evolutionary dynamics is important to correctly assess evo-to-eco feedbacks. For example, it is assumed that multiple species will evolve, yet empirical evidence on the ecological consequences of multi-species evolution is limitedly studied in nature, nor in the context of urbanization.

While freshwater ecosystems such as ponds and pools are crucial for regional biodiversity, research on these ecosystems in urban ecology, but more specifically urban evolution, was very limited. My PhD contributed to the fast growing evidence of urbanization driven evolution, already observed in terrestrial ecosystem. The results presented in this dissertation revealed striking patterns of multi-trait evolution in not less than 14 out of 17 measured traits in the key interactor, *Daphnia magna*, including syndrome structures. We propose future studies to address the possible consequences of such urban adaptations for local and regional ecological processes, with a priority for ecosystem functions (stabilizing the clear-water phase) and range expansion dynamics under climate change.

Samenvatting

Verstedelijking veroorzaakt omgevingsveranderingen die tot snelle evolutie kunnen leiden. Een belangrijk kenmerk van stedelijke gebieden is hun hogere temperatuur (i.e. het 'stedelijk hitte-eiland effect'). Over de invloed van dergelijke antropogene opwarming op kleine zoetwaterecosystemen, zoals vijvers, en de organismen die er in voor komen, is tot nog toe weinig gekend.

We monitorden de watertemperatuur in stedelijke en landelijke vijvers in Vlaanderen (België, jaarlange studie) en konden vaststellen dat stedelijke vijvers aanzienlijk warmer zijn (minimum, maximum, gemiddelde temperaturen) en gekenmerkt zijn door een langer geschat groeiseizoen. We voerden common garden experimenten uit in het laboratorium om te testen of gerepliceerde stedelijke en landelijke populaties van de watervlo *Daphnia magna* (13 populaties in totaal) genetische verschillen voor een reeks kenmerken waaronder hittetolerantie (i.e. het kritisch thermaal maximum, of KT_{MAX}), levensgeschiedeniskenmerken (leeftijd van maturiteit, grootte bij maturiteit, ontwikkelingstijd, intrinsieke populatiegroeisnelheid, somatische groei) en stressfysiologie (concentratie van vet, eiwitten, suikers, anti-oxidatieve afweer via enzymatische activiteit, oxidatieve schade, hemoglobineconcentratie).

In vergelijking met rurale populaties hebben stedelijke *Daphnia* populaties een genetisch hogere hittetolerantie (tot 2 °C) en een snellere levensstijl. Multivariate fenotypische trajectanalyses wijzen op een genetische differentiatie van levensgeschiedeniskenmerken langsheen de trage-naar-snelle as van het zogenaamde 'levensritme' continuüm (i.e. 'pace-of-life'). Urbane dieren matureren vroeger en hebben een kortere lichaamslengte bij maturiteit (in lijn met de temperatuur-grootte regel), een hogere vruchtbaarheid en een hogere intrinsieke populatiegroeisnelheid. Bovendien tonen stressfysiologische analyses aan dat stedelijke dieren een hoger concentratie energie reserves hebben (hogere concentraties vetten, eiwitten en suikers). Hoewel urbane watervlooien een lagere activiteit van anti-oxidante enzymen vertonen, vonden we opmerkelijk dat lipidenperoxidatie (oxidatieve schade) niet verschilde tussen de twee genotypesets, wat suggereert dat stedelijke populaties van *Daphnia* een hogere stress tolerantie hebben. Bovendien zijn alle levensgeschiedenis- en fysiologische kenmerken significant gestructureerd door een onderliggend pace-of-life syndroom (POLS) in de stedelijke genotypesets, maar niet in de landelijke. We veronderstellen dat de gecombineerde mix van stressoren (temperatuur, vervuiling, vijverbeheer) ervaren door dieren in stadsvijvers de toewijzing van energie zou kunnen verschuiven om stressfysiologische tolerantiemechanismen in overeenstemming te brengen met de levensgeschiedenisevolutie in de richting van een kortere lichaamslengte en sneller levensritme, resulterend in een sterke POLS. In de rurale gebieden kan energie potentieel meer geïnvesteerd worden in, bijvoorbeeld, anti-predatie verdedigingsmechanismen of immunologische responses. Samen geven deze resultaten aan dat verstedelijking de evolutie van hittetolerantie, levenssnelheid, energie reserves, stresstolerantie en het optreden van een pace-of-life syndroom kan beïnvloeden in watervlo populaties.

Analyse van zoöplankton gemeensschapsdata, een integratie van relatieve abundanties en soortspecifieke lichaamslengtemetingen langs een verstedelijkingsgradiënt bestaande uit 83 vijvers, duidde op een vergelijkbare afname van de gemiddelde lichaamsgrootte van de zoöplanktongemeenschap met toenemende verstedelijking. Dit geeft aan dat evolutionaire en ecologische kenmerkverschuivingen in respons op verstedelijking opmerkelijk vergelijkbaar zijn en in beide gevallen een 'verkleining' van het zoöplankton aangeven. Deze reductie in lichaamslengte is hoogstwaarschijnlijk het resultaat van de verhoogde temperaturen in de stedelijke vijvers. Toch is het niet onwaarschijnlijk dat evolutionaire en ecologische responsen ook door andere omgevingsverstoringen zoals eutrofiëring, vervuiling of veranderingen in biotische interacties (predatie, gastheer-parasiet interacties) worden gedreven..

Om een mogelijk effect van verstedelijkingsgedreven evolutie van meerdere soorten op ecologische dynamiek ('eco-evolutionaire feedback') te testen, voerden we een groot common garden experiment met zowel stedelijke als rurale watervlooien (*Daphnia magna)* en waterjuffer larven (*Ischura elegans*, een veel voorkomende predator van de watervlo). Predatoren en prooien werden aan elkaar blootgesteld in milde tot intense, gesimuleerde, hittegolven in het laboratorium. Daarbij werd de evolutionaire (stedelijke) achtergrond van de predator en de prooi reciprook gekruist (i.e. stedelijk-stedelijk, stedelijk-landelijk, landelijk-stedelijk, landelijk-landelijk). De resultaten wijzen op een (asymmetrisch) 'mismatch'-effect: terwijl stedelijke predatoren een hogere predatie vertonen in vergelijking tot rurale predatoren wanneer ze blootgesteld worden aan rurale *Daphnia*, verdwijnt dit predatie verschil wanneer ze worden blootgesteld aan stedelijke watervlooien. Dit geeft aan dat de stedelijke en dus evolutionaire achtergrond van zowel de predator als de prooi het resultaat van hun interactie vormen (i.e. predatie efficiëntie). Deze resultaten zijn een voorbeeld van cryptische eco-evolutionaire dynamieken, waarbij evolutie in de ene soort het ecologisch effect van evolutie in de andere soort buffert. We veronderstellen dat de signalen van thermische aanpassing, die eerder waargenomen zijn bij zowel predator als prooi, de belangrijkste oorzaken zijn van dit 'mismatch'-effect. Het waarnemen van zulke cryptische eco-evolutionaire dynamieken is belangrijk om eco-evolutionaire dynamieken en feed backs correct te bestuderen. Bijvoorbeeld, er wordt verondersteld dat meerder soorten in de natuur evolutie kunnen vertonen, maar de ecologische gevolgen van evolutie in meerdere interagerende soorten is nog niet vaak bestudeerd in de natuur, noch in de context van verstedelijking.

Hoewel zoetwater ecosystemen zoals vijvers en poelen cruciaal zijn voor regionale biodiversiteit, was het onderzoek op deze ecosystemen in stadsecologie en meer specifiek evolutiebiologie in de stad uiterst beperkt. Mijn doctoraat droeg bij tot het snelgroeiend bewijs van verstedelijkingsgedreven evolutie, zoals waargenomen in terrestrische ecosystemen, en onthulde opvallende patronen van multi-kenmerk-evolutie in niet minder dan 14 van de 17 gemeten kenmerken in één van de belangrijkste watervlo soorten, *Daphnia magna*. Toekomstige studies zullen moeten uitwijzen wat de mogelijke gevolgen van dergelijke stedelijke aanpassingen zijn voor lokale en

regionale ecologische processen, onder meer ecosysteemfuncties (stabilisatie van de heldere water toestand) en adaptatie aan klimaatsveranderingen.

Publications

Publications

Brans, K. I., Stoks, R., & De Meester, L. (2018b). Urbanization drives genetic differentiation in physiology and structures the evolution of pace-of-life syndromes in the water flea *Daphnia magna*. *Proceedings of the Royal Society B- Biological Sciences*, 285(1883), 20180169. doi:10.1111/1365-2435.13184

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