# EFFECTS OF URBANISATION ON THE ECOLOGY AND EVOLUTION OF A DAMSELFLY

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## TABLE OF CONTENTS

# GENERAL INTRODUCTION

Humans have modified nearly all environments they have occupied, changing the dynamics of many ecosystems on Earth (Vitousek et al. 1997; Kaye et al. 2006). One such anthropogenic process, the ongoing rapid pace of urbanisation (Seto et al. 2012; UN 2014) causes tremendous impact on the environment, including increased temperatures and pollution, habitat fragmentation and destruction, and altered noise and light regimes (Parris 2016). Although efforts to understand how urbanisation influences the biotic environment mainly focused on species abundances and biodiversity (e.g. McIntyre 2000; Alberti and Marzluff 2004; McKinney 2006, 2008; Shochat et al. 2006; Willigalla and Fartmann 2012; Martinson and Raupp 2013; Dale and Frank 2014; Youngsteadt et al. 2015; Concepción et al. 2015, 2017; Iwai et al. 2017; Sol et al. 2017; Lososová et al. 2018; Nguyen et al. 2018), it is important to understand that these influences are mediated through organismal traits such as physiology and behaviour (Chown and Duffy 2015). This triggered a call for a more mechanistic approach in urban ecology research (Shochat et al. 2006; Sol et al. 2013; Alberti 2015). The recent decade has witnessed an explosion of studies demonstrating not only plastic but also evolutionary responses to urbanisation in a wide range of organisms (reviewed in Neil and Wu 2006; Donihue and Lambert 2015; Alberti 2015; Hendry et al. 2017; Alberti et al. 2017a; Johnson and Munshi-South 2017; Sullivan et al. 2017; Schell 2018). Yet, our understanding of how urbanisation drives evolution is still in its infancy (Johnson and Munshi-South 2017). Moreover, urbanisation-driven evolutionary changes in phenotypes may generate feed-back loops from evolution to ecology, hence shaping eco-evolutionary dynamics (Alberti 2015; Alberti et al. 2017b; Hendry et al. 2017; Schell 2018). Interestingly, current conditions in cities often resemble those expected under the ongoing global change in the near future, making urban areas potential “laboratory” systems to predict species responses to anthropogenic impact, e.g. to climate change (Farrell et al. 2015; Jochner and Menzel 2015; Chown and Duffy 2015; Youngsteadt et al. 2015; Youngsteadt et al. 2017). In this thesis, I studied the effects of urbanisation on a semiaquatic model system, the damselfly, with an emphasis on life-stage specific responses to urban-stressors.

I will start this chapter by introducing the phenomenon of urbanisation, and describe relevant stressors associated with urban areas. Next, I will give an overview of documented species responses to urban stressors, followed by a section where I highlight several gaps in the field. Following a description of the study species and the selection of study populations, I will describe the focal traits I used in my studies. Finally, I will give a description of the general aims of my PhD project, and outline experimental approaches applied in my studies.

## Urbanisation and associated stressors

Currently more than half of the human population lives in urban areas, and predictions indicate this will exceed 60% by 2030 (Grimm et al. 2008b; UN 2014). Given that this number was ca. 10% in 1900, urbanisation can be classified as a rapid anthropogenic process operating on a global scale. The ongoing urbanisation has caused strong changes in ecosystems (Grim et al. 2008a) by altering land use, biodiversity, and biogeochemical cycles (Grimm et al. 2008b; Kaye et al. 2006). Aside from the three focal stressors discussed in detail below, anthropogenic disturbances associated with urbanisation include stressors such as altered food sources (e.g.; Ditchkoff et al. 2006; Murray et al. 2015; Snell-Rood et al. 2015), predation pressure (e.g. Gering and Blair 1999; Jokimäki and Huhta 2000) and prey abundance (e.g. Teglhøj 2017), increased light (Gaston et al. 2015; Knop et al. 2017) and noise pollution (Katti and Warren 2004; Slabbekoorn 2013). These characteristics suggest urban areas to be more unpredictable in their nature compared to natural rural habitats (Parris 2016), and distinct differential selection pressures on organismal traits can be expected in urban compared to rural areas, with implications for fitness and adaptation (Shochat et al. 2006).

### *(i) Temperature*

Arguably, the most well-known stressor associated with urbanisation is the higher temperatures encountered in the city than in natural areas. This so called urban heat island (UHI) effect is the result of a combination of several processes typically encountered in cities (Oke 1973; Arnfield 2003): high thermal conductivity of buildings, impervious surfaces with low albedo (i.e. surface reflectivity) and high heat capacity (Taha 1997), low abundance of vegetation (Jochner and Menzel 2015) causing reduced evaporative cooling (Taha et al. 1991), and heat output by motor vehicles and air-conditioners (Fan and Sailor 2005). The UHI effect represents one of the most significant anthropogenic influences on the Earth's surface climate (Kalnay and Cai 2003; Zhou et al. 2004; Zhao et al. 2014), and can drive temperature differences between urban and the surrounding rural areas from a moderate 1 °C to as high as 10 °C (e.g. Kim 1992; Rosenzweig et al. 2009; Imhoff et al. 2010; De Ridder et al. 2015; Kaiser et al. 2016). Although few have studied the UHI effect on water temperatures (but see Kaushal et al. 2010; Hester and Bauman 2013; Somers et al. 2013; Brans et al. 2018), a similar effect can be expected given the obvious strong correlation of air and water temperatures (e.g. see Figure 2. in Rollinson and Rowe 2018). The higher potential of runoff in urban compared to rural areas can further contribute to the higher temperatures in urban water bodies (Herb et al. 2008; Hester and Bauman 2013; Somers et al. 2013). In Flanders (Belgium), the study region of this PhD project, summer average water temperatures are ca. 3 °C higher in urban than rural ponds, and the daily maximum temperature difference is as high as 3.7 °C (Brans et al. 2018).

The UHI effect may alter the available time for organisms to grow; i.e. the length of the growing season. Reports for extended growing season due to the UHI effect are common (e.g. Zhang et al. 2004; Yang et al. 2013; Zipper et al. 2016; Li et al. 2017), where suitable conditions for growth arrive earlier and end later in urban areas, resulting in a longer growing season. As will be discussed later in detail, changes in growing season length strongly shape phenology; i.e. the timing of life cycle events. Altered plant phenologies are even suggested as a tool for monitoring the UHI effect in cities (Jochner et al. 2013).

Next to causing higher average temperatures, the increased runoff in urban areas can also result in highly variable water temperatures over short time scales (Wenger et al. 2009). While the intensity, frequency, and duration of heat waves are predicted to increase with global warming (Meehle and Tebaldi 2004), the UHI effect is expected to further exacerbate the heat stress in cities during heat wave episodes (Li and Bou-Zeid 2013; Ward et al. 2016; Wouters et al. 2017; Zhao et al. 2018). The less abundant vegetation in urban areas (Jochner and Menzel 2015) may cause a less efficient buffering of daily temperature variations in ponds via shading and evapotranspiration (Taha et al. 1991; Yu and Hien 2006; Alavipanah et al. 2015). Indeed, summer daily temperatures in urban ponds fluctuated ca. 2 °C more compared to rural ponds in the study region (Brans et al. 2018; see Kaiser et al. 2016 for a similar pattern in air temperature).

#### *(ii) Pollution*

Air, water and soil pollution are other stressors associated with urban areas (Parris 2016). For example, increased metal pollution in urban areas (e.g. Gorissen et al. 2005; Roux and Marra 2007) can be detected in feathers (Eens et al. 1999), eggs (Burger et al. 2004) and blood (Roux and Marra 2007) of birds inhabiting cities. Similarly, water bodies in urban areas are often more polluted when compared to surrounding natural water bodies, including higher concentrations of metals, pharmaceuticals, and pesticides (Paul and Meyer 2001; Walsh et al. 2005; Metcalfe et al. 2010; Hayzoun et al. 2014), mainly due to increased use of chemicals in urban settlements. The high percentage of impervious surfaces in cities further contributes to the higher pollution load in water bodies due to increased runoff (Paul and Meyer 2001; Beasley and Kneale 2002; Qin et al. 2010; Jiang et al. 2010; Thuyet et al. 2012; Hassall 2014; Young et al. 2018). Especially pesticides have been found in higher concentrations in urban water bodies (Hoffman et al 2000; Paul and Meyer 2001; Gilliom 2007; Wenger et al. 2009; Stone et al. 2014; Wang et al. 2016), often exceeding guidelines for the protection of aquatic biota (Hoffman et al. 2000; Paul and Meyer 2001).

#### *(iii) Habitat fragmentation*

The intense conversion of natural areas into urban land results in fragmented natural habitats (Marzluff 2001; Luck and Wu 2002; Cane et al. 2006; Parris 2016). This habitat modification due to urbanisation results in smaller and more isolated populations (Andren and Delin, 1994; Moilanen and Nieminen, 2002), as suitable habitat patches in urban areas are typically disconnected from each other due to urbanisation eliminating crucial corridors (Alberti and Marzluff 2004; Grimm et al. 2008a). For example, odonate connectivity has been shown to be reduced in fragmented city habitats that lack corridors to facilitate dispersal (Watts et al. 2004; Sato et al. 2008). Roads can contribute to this urbanisation-driven habitat fragmentation (Forman and Alexander 1998), whereas the heavy traffic on city roads can further act as strong barriers to the movement of many organisms (e.g. for flying insects: Muñoz et al. 2015). As in terrestrial habitats, waterbodies in urban areas have a decreased connectivity to other waterbodies (Hassall 2014).

### Responses to urban stressors

A large body of research has been conducted on the species responses to urbanisation, which have been assembled, mainly in the last decade, in the form of reviews (Chamberlain et al. 2009; Alberti et al. 2015; McDonnel and Hahs 2015; Alberti et al. 2017b; Johnson and Munshi-South 2017; Sepp et al. 2018) and meta-analyses (Chamberlain et al. 2009; Martinson and Raupp 2013; Alberti et al. 2017a; Sepp et al. 2018). Yet, ignoring the constituents of the urbanisation phenomenon does not aid in understanding the mechanisms underlying responses to this anthropogenic process. It is the individual processes associated with urbanisation that cause ecological alterations, hence identifying these stressors is a necessary step in gaining insight into the organismal responses to urbanisation. This point becomes especially relevant when considering that responses to particular urban-related stressors will be highly species-specific (e.g. Beninde et al. 2015). For example, increased exposure to humans is suggested as the main cause for the apparent decreased shyness in urban populations of several bird species (e.g. Miranda et al. 2013; Sol et al. 2013; Samia et al. 2015), whereas for insects, this reasoning can hardly be relevant (see also Schuett et al. 2018).

### *(i) Temperature*

Even small changes in temperatures can dramatically influence the fitness of organisms (Deutsch et al. 2008; Angilletta 2009). These fitness effects operate via temperature-induced responses of traits such as life-history, behaviour, morphology and physiology (Angilletta 2009; for odonates: Hassall and Thompson 2008). Plastic and genetic responses to the UHI effect have been documented for many different taxa (reviewed in Chown and Duffy 2015). The most obvious adaptive response to the higher temperatures experienced in cities is a higher heat tolerance. Indeed, the few existing studies reveal a higher heat tolerance in urban compared to rural populations of ants (leafcutter ants: Angilletta et al. 2007; acorn ants: Diamond et al. 2017) and the waterflea *Daphnia magna* (Brans et al. 2017b). Another predicted life-history response to UHIs is smaller body size (Merckx et al. in press) due to increased temperatures (i.e. the temperature-size rule, Atkinson 1994), as was documented for *D. magna* (Brans et al. 2017a; Brans et al. 2018), yet this rule may strongly depend on the growth and development rates (Walters and Hassall 2006), which themselves are influenced by the UHI-induced changes in growing season length (discussed below). For example, males of the butterfly *Lasiommata megera* that developed in urban sites tended to have larger body sizes, driven by the higher urban temperatures (Kaiser et al. 2016).

The direct effect of UHIs on growth and development rates are not clear. Urban populations of the grasshopper *Chorthippus brunneus* showed faster growth rates, yet did not differ in development time when compared with rural populations (San Martin y Gomez and Van Dyck 2012). In the only study with an aquatic ectotherm, urban clones of the water flea *Daphnia magna* have evolved a shorter development time than rural clones (Brans et al. in review). Thermal performance curves, continuous reaction norms of organismal performance traits (e.g. growth rate) in response to temperature (Huey and Stevenson 1979; Izem and Kingsolver 2005), are ideal tools to study differentiation in thermal responses (Stinchcombe and Kirkpatrick 2012; Sinclair et al. 2016). Surprisingly, this approach was only once applied to test differentiation in thermal responses of urban and rural populations: some urban fungal isolates showed higher growth rates than rural isolates at higher temperatures, whereas other urban isolates had similar or faster growth at all temperatures compared with rural isolates (McLean et al. 2005).

The UHI effect may also have an indirect effect on organisms via altering growing seasons. Extended growing season lengths due to the UHI effect (e.g. Zhang et al. 2004; Yang et al. 2013; Zipper et al. 2016; Li et al. 2016), and the corresponding shift in phenology has been well documented for plants (e.g. Zhang et al. 2004; Neil and Wu 2006; Jochner and Menzel 2015; Li et al. 2017). Studies on the effects of urbanisation on animal phenology are mainly based on birds, where a frequent finding is an advancement in the timing of seasonal reproduction in the urban inhabitants (reviewed in Chamberlain et al. 2009; Deviche and Davies 2014; see also Charmantier et al. 2017). Yet, this response is often attributed to factors other than the UHI (e.g. food availability; Chamberlain et al. 2009). Other terrestrial vertebrates also show advanced reproductive phenology in urban populations due to the UHI (e.g. lizards: Hall and Warner 2017; salamander: Cook et al. 2006). The phenology of insects in general seems to be positively affected by climate change (Bale et al. 2002; for odonates: Hassall et al. 2007), therefore a similar pattern can be expected for the higher urban temperatures. Yet, very few studies have investigated this. Mosquitoes indeed seem to follow the expected pattern, where advanced phenology in urban areas was reported (Townroe and Callaghan 2014). Patterns of phenological responses to urbanisation in a set of butterfly species were more complicated, and strongly dependent on geographic variation in temperature (Diamond et al. 2014). Interestingly, another study with butterflies found delayed phenologies in urban areas, possibly due to dispersal among habitat types causing a source-sink effect (Altermatt 2012). Finally, the UHI was found to advance odonate phenology, although this effect was quite subtle when compared to the effect of climate change (Villalobos-Jiménez and Hassall 2017).

Survival and reproductive success, two key fitness traits, can also be altered by the UHI. The higher abundance, possibly due to higher survival, of the scale insect *Parthenolecanium quercifex* was suggested as an adaptation to the UHI (Meineke et al. 2013). Larval survival of the butterfly *Lasiommata megera* was higher in urban compared to rural sites (Kaiser et al. 2016). The UHI (together with increased prey availability) potentially caused the increased fecundity in urban population of the orb-weaving spider *Nephila plumipes* (Lowe et al. 2014). Higher temperatures due to UHI significantly increased scale insect fecundity (Dale and Frank 2014). Studies with passerine birds suggest a lower productivity in urban populations (Chamberlain et al. 2009), which could be due to reduced clutch size (operating via earlier breeding times in cities) and a reduced nestling body mass in urban populations (Sprau et al. 2017).

### *(ii) Pollution*

Apart from causing direct mortality, exposure to contaminants can have sublethal effects on organisms (Köhler and Triebskorn 2013; for damselflies: Stoks et al. 2015). Behaviour is a trait well known to respond to contaminant exposure (Dell'Omo 2002; Zala and Penn 2004; Tuomainen and Candolin 2011). Sublethal contaminants often alter fitness-related behaviours such as activity (e.g. Jonsson et al. 2014; Hansen and Roslev 2016), boldness (e.g. Brodin et al. 2013; Debecker et al. 2016), exploration (e.g. Morales et al. 2013; Dzieweczynski et al. 2018) and food intake (e.g. Dinh Van 2013; Zubrod et al. 2014). Aside from mean levels of behaviours, concepts more familiar to behavioural ecologists such as personality, repeatability, and behavioural syndromes (Sih et al. 2004b; Réale et al. 2007) have recently entered the field of ecotoxicology (Montiglio and Royauté 2014). Personality is defined as individual differences in behaviour, and is often said to exist when repeatable differences in behaviour among individuals (i.e. repeatability) is detected (Réale et al. 2007). An increased exposure to a contaminant driven by the personality of the individual, e.g. more active, may feed-back negatively via the toxicity of the contaminant, decreasing the expression level of the behaviour in question. This would weaken individual differences in behaviour, leading to a lower repeatability. Studies indeed found decreased repeatability in behaviours when animals had been exposed to a contaminant (Kolok et al. 1998; Dzieweczynski, 2011; Royauté et al. 2015; Dzieweczynski et al. 2016; but see Tosetto et al. 2017; White and Briffa, 2017). Similarly, behavioural syndromes (consistent correlations among behaviours) are often found to be influenced by contaminant exposure (Brodin et al. 2013; Royauté et al. 2015; Dzieweczynski et al. 2016). It is suggested that certain combinations of behaviours are selected for as a result of different selection pressures (Bell 2005; Sih et al. 2004a; Sih et al. 2012). The appearance of correlations between previously uncorrelated behaviours may be the result of stronger selection pressures selecting for tighter behavioural correlations (e.g. Bell and Sih 2007; Snekser et al. 2009), hence a similar effect might be expected after contaminant exposure (Brodin et al. 2013). Yet, disappearance of behavioural correlations following contaminant exposure has also been observed (Royauté et al. 2015; Dzieweczynski et al. 2016), possibly because the toxicity may decouple the correlations via physiological alterations.

Life history traits also respond to contaminant exposure. This is often mediated via reductions in resource uptake and/or changes in resource allocation whereby exposure can result in diversion of energy from somatic maintenance to detoxification and repair (Sibly and Calow 1989;

Congdon et al. 2001). As a result, many studies reported a reduction in growth rate due to exposure to contaminants (e.g. Arambourou and Stoks 2015; Op de Beeck et al. 2017).

Populations experiencing higher contaminant levels may evolve a lower vulnerability to these contaminants (reviewed in Medina et al. 2007; but see e.g. Crespi 2000; Spitzer 2006). Yet, we currently do not know the extent of local adaptation of urban populations to better deal with contaminants compared to rural ones. One exception is the detailed work done on the killifish *Fundulus heteroclitus*, where urban populations in polluted waters have evolved higher tolerance to polychlorinated biphenyls (Whitehead et al. 2012; Reid et al. 2016). This was linked to modifications in the aryl hydrocarbon receptor–based signalling pathway (Whitehead et al. 2010; Reid et al. 2016).

***(iii) Habitat fragmentation***

Fragmentation often causes physical isolation of habitat patches, decreasing the structural connectivity of a landscape, which in turn can hinder movement between patches and alter dispersal patterns (Henle et al. 2004; Fischer and Lindenmayer 2007; Concepción et al. 2015; Evans et al. 2017). Fragmented landscapes are expected to select for traits facilitating easier movement between isolated habitat patches (Van Dyck and Matthysen 1999; Dover and Settele 2009). Dispersal ability is a relevant trait to study in this context (LaPoint et al. 2015), given that populations in fragmented habitats are often found to have evolved increased dispersal abilities (Van Dyck and Matthysen 1999; Baguette et al. 2012; Ducatez et al. 2013; Cote et al. 2017; but see Bergerot et al. 2012).

Human-driven fragmentation is known to have a strong impact on abundance and species richness and species composition of communities (Laakkonen et al. 2001; Maes and Van Dyck 2001; Tscharntke et al. 2002; Fahrig 2003), and more general on ecosystems (Haddad et al. 2015). For example, bee abundance and species richness are reduced due to anthropogenic habitat fragmentation (Winfree et al. 2009; but see Cane et al. 2006 for less clear patterns). Selection pressures of urban fragmentation causing such shifts in community composition are likely to act on functional traits (Violle et al. 2007; Webb et al. 2010). Indeed, urban communities of carabid beetles consisted of species with better dispersal capacities compared with urban communities (Piano et al. 2017). Besides species sorting, also sorting of genotypes within species based on functional traits is expected to occur along urbanisation gradients. Yet, no studies looked at the latter evolutionary processes on dispersal ability.

Although it is known that organisms can show adaptations to anthropogenic fragmentation (Cheptou et al. 2017), few studies considered differentiation in phenotypic traits associated with habitat fragmentation and isolation along urbanisation gradients (but see e.g. Cheptou et al. 2008; Dubois and Cheptou 2017 for plants). Given the typically fragmented and isolated status of urban habitats (Marzluff 2001; Luck and Wu 2002; Cane et al. 2006; Parris 2016), the expectation of an increased dispersal ability in urban populations resembles the selection forces experienced at invasion fronts of range expanding species. Indeed, the more recently colonised edge populations often show increased dispersal ability when compared with core populations (Hill et al. 2011; Chuang and Peterson 2016). For example, cane toads at the invasion front evolved a higher locomotor ability (Shine 2012), and flight endurance was reported to be higher at the range expansion front in the damselfly *Coenagrion scitulum* (Swaegers et al. 2015). Both spatial sorting (Shine et al. 2011), the process where only the organisms with the best locomotor abilities end up at the range front, and local adaptation (Travis and Dytham 2002) may contribute to dispersal-enhancing phenotypes at invasion fronts, although spatial sorting has been suggested as the main driver (Van Petegem et al. 2016). Following this analogy between urban and edge populations, only the best dispersers from the rural populations are expected to enter and colonise urban habitats. Moreover, colonisation of empty patches is another process tightly associated with dispersal ability. Given the more fragmented habitats in urban areas, and that urban populations are expected to be colonised from rural source populations (e.g. Evans et al. 2009, 2012; Stracey and Robinson 2012), a higher dispersal ability is predicted in populations inhabiting more fragmented urban areas. The few studies on insects comparing locomotor ability between urban and rural populations indeed found larger wings in urban compared to rural populations (the grasshopper *Chorthippus brunneus*: San Martin y Gomez and Van Dyck 2012, the butterfly *Pieris rapae*: Schoville et al. 2013), which was interpreted as selection for increased mobility.

### Knowledge gaps in the field

Despite the abundance of studies on responses to urbanisation, several important aspects have received much less attention. Each of these aspects, described below, will be addressed in one or more chapters of this thesis.

### *(i) Effects of urbanisation on (semi)aquatic animals*

The majority of studies on the effects of urbanisation have concentrated on birds and mammals (Magle et al. 2012; Beninde et al. 2015). Aquatic animals are clearly underrepresented in the study of urbanisation effects, despite their sensitivity to urban stressors (e.g. aquatic invertebrates: Paul and Meyer 2001). This is worrying, as water bodies in urban areas are as much affected by urban stressors as terrestrial habitats (Hassall 2014), if not more, given the additional runoff effect in cities (for pollution: e.g. Paul and Meyer 2001; temperature: e.g. Somers et al. 2013). Studying the effects of UHIs may be especially relevant in aquatic systems, as higher temperatures often increase the oxygen demand of animals, whereas higher water temperatures result in a limited availability of oxygen (Bozinovic and Pörtner, 2015; Chown et al. 2015; Horne et al. 2015). This is relevant for aquatic ectotherms, which seem to be particularly sensitive to elevated temperatures (e.g. Verberk et al. 2016). Moreover, chemical pollution as an anthropogenic environmental stress is especially prevalent in aquatic environments (Schwarzenbach et al. 2006). Aquatic animals are in a disadvantage when it comes to avoiding contaminant exposure in waterbodies, given their limited escape possibilities (e.g. damselflies: Stoks et al. 2015). Hence, aquatic invertebrates are excellent study species for research on ecotoxicology and urban adaptation.

### *(ii) Carryover effects of urban-related stressors*

Conditions experienced during the early phases of development can have long lasting effects that can persist throughout the life of the individual (Monaghan 2008). This becomes especially relevant for species that undergo major changes in their body plan (i.e. metamorphosis), as well as experience a drastic habitat shift (e.g. aquatic vs terrestrial) during their ontogeny. Species with such “complex life cycles”, which includes the majority of animals (Wilbur 1980; Werner and Gilliam 1984), are attractive study systems for carryover effects. The metamorphosis events usually consist of a dramatic change and reorganization of the body plan (i.e. morphology and physiology), hence it can be expected that the effect of a stressor experienced early in life will not be influencing the adult stage in the same manner as it did in the earlier (e.g. larval) stage (Wilbur 1980; Moran 1994). Further, the aquatic larvae of a species with a complex life cycle are exposed to clearly distinct environmental conditions (stressors) when compared to the conditions experienced in the terrestrial adult stage (e.g. odonates: Stoks and Córdoba-Aguilar 2012). Importantly, carry-over effects of stressors are assumed to operate via metamorphic traits (i.e. age and mass at metamorphosis; Earl and Whiteman 2015), yet several findings have casted doubt on the reliability of this assumed pathway (De Block and Stoks 2005; Tigreros 2013; Earl and Whiteman 2015; Rosa and Saastamoinen 2017). Considering the link between life stages is crucial for a comprehensive understanding of the responses to environmental stressors (e.g. Iwai et al. 2017). To my knowledge, no study has tested whether urban and rural populations differ in the way effects of stressors experienced early life carry over and influence later life stages.

### *(iii) Urbanisation and dispersal*

There is a clear gap in our understanding how urbanisation-driven habitat fragmentation influences dispersal and dispersal-related traits in animals (LaPoint et al. 2015; but see e.g. Cheptou et al. 2008; Dubois and Cheptou 2016 for plant studies). This is surprising, given the tight link between urbanisation and habitat fragmentation (Luck and Wu 2002; Cane et al. 2006; Parris 2016), and the well-established relationship between dispersal ability and habitat fragmentation (Van Dyck and Matthysen 1999; Baguette et al. 2012; Cote et al. 2017). Fragmentation often causes physical isolation of habitat patches, decreasing the structural connectivity of a landscape, which in turn can hinder movement between patches and alter dispersal patterns (Fischer and Lindenmayer 2007). Several findings indicate the potential for micro-evolution of dispersal and dispersal-related traits (Saastamoinen et al. 2018), driven mostly by range expansion processes, or by colonisation of fragmented habitats. A recent study reports that higher dispersal ability can be advantageous for survival in urban habitats and other highly fragmented habitats (Rochat et al. 2017). To gain better insight into how urbanisation might influence population persistence of insects in urban areas, more research on adult dispersal is required (Smith et al. 2009).

### *(iv) Urbanisation and sexual selection*

Sexual selection can aid in facilitating adaptation to anthropogenic environments (Candolin and Heuschele 2008), whereas anthropogenic environments themselves can shape sexual selection (e.g. Candolin et al. 2007; Lane et al. 2011). Several studies investigated urbanisation-driven changes in sexually selected traits. For example, human-induced habitat fragmentation has caused a divergence in sexually selected traits of *Gambusia* fish such as fin coloration and genitalia size (Heinen-Kay et al. 2014; Giery et al. 2015). Other than direct effects of urbanisation on sexual selection regimes, urbanisation-driven differences in mean phenotypic traits between populations may also alter the direction and strength of selection: populations that differ in their frequency distribution for a given trait will occupy different parts of the species' fitness function, which in turn may cause different selection patterns in the different populations (in case of a non-linear fitness function; Endler 1986; Conner and Hartl 2004). Given that urbanisation has the potential to drive changes in mean phenotypic traits of organisms (e.g. Alberti et al. 2017a), different selection regimes between urban and rural populations can be expected. Yet, studies explicitly measuring sexual selection along urbanisation gradients are rare (Johnson and Munshi-South 2017).

### *(v) Interactive urban stressors*

Stressors influencing organisms in urban areas do not act in isolation, but rather do so simultaneously and in a dynamic manner (Ramalho and Hobbs 2012; Galic et al. 2018). Multistressor studies often show interactive effects, where one treatments' influence depends on the other treatment. Given that stressors associated with urbanisation, e.g. pollution and warming, are often used as treatments in such studies, it is crucial to acknowledge potentially interactive effects at play in urban ecosystems (Ramalho and Hobbs 2012). Climate change and urbanisation, for example, can interactively shape aquatic ecosystems (Nelson et al. 2009; Radinger et al. 2016) and butterfly phenology (Diamond et al. 2014). Habitat fragmentation and climate change has also been shown to have interactive effects on biodiversity (Opdam and Wascher 2004).

## The study species

To address these knowledge gaps, I used damselflies as model organisms. Damselflies are suitable model organisms to study evolutionary and plastic responses to human-induced stressors such as climate change (Hassall and Thompson 2008; Hassall 2015) and contaminants (Stoks et al. 2015), as well as urbanisation (Villalobos-Jiménez et al. 2016). They strongly react to time stress (e.g. Stoks et al. 2012; Śniegula et al. 2016). Importantly, given their complex life cycle with an aquatic larval stage followed by the terrestrial adult stage, damselflies are expected to be under distinct selection pressures at different life stages (Stoks and Cordoba 2012), including to urban-stressors (Villalobos-Jiménez et al. 2016). Because of this, damselflies are also ideal systems to study carry-over effects, for example how stressors experienced during larval stage influence the adult phenotype, and how this might affect food webs (damselfly larvae are intermediate predators) across ecosystem boundaries (Knight et al. 2005; Greig et al. 2012; Kraus et al. 2014). Damselflies exhibit personality and behavioural syndromes (Debecker et al. 2016), which can be consistent across metamorphosis (Brodin 2009). Moreover, the study of pace of life syndromes, i.e. the integration of various traits that have coevolved along a fast-slow continuum (Ricklefs and Wikelski 2002; Réale et al. 2010) has also been successfully applied to damselflies (Debecker et al. 2016). Lastly, because reproduction occurs at water bodies, damselflies are ideal organisms to study sexual selection in natural populations.

As study species, I chose the damselfly *Coenagrion puella*. This species is abundant both in urban and rural ponds (Goertzen and Suhling 2013; Willigalla and Fartmann 2012; Le Gall et al. 2018). The species has been well studied in terms of life-history, behaviour and reproductive biology (e.g. Banks and Thompson 1985b; Thompson 1990; Lowe et al. 2009; Thompson et al. 2011; Gyulavári et al. 2014). At the latitude of the study region, *C. puella* is univoltine (Corbet et al. 2006).

**Selection of study populations**

For all experiment presented in this thesis, I used the same set of three rural and three urban damselfly populations. The populations were within a 45 km radius in Flanders, Belgium. Populations were categorized as “rural” or “urban” based on the % built-up area using GIS data (following Merckx et al. in press). Rural populations were surrounded by <3% built-up area and urban populations with >15% built-up area. This categorization was based on a two-step procedure. First, I selected three rural and three urban plots of 3 × 3 km. Within these plots, I selected a subplot of 200 × 200 m with the same urbanisation level. This method ensured that the assigned urbanisation level (i.e. rural or urban) was valid at both the local and regional level (see also Kaiser et al. 2016; Piano et al. 2017). This is a necessary step for the simplification of the experimental design, as different mechanisms may operate at different spatial scales (Clergeau et al. 2006), and the scale at which urbanisation is assessed proved to be an important explanatory factor when studying responses to urbanisation (Kaiser et al. 2016; Piano et al. 2017; Brans 2017a; Dahirel et al. 2017).

The approach of categorizing rural and urban populations followed in this study, i.e. % built-up area, indeed seem to represent the here defined stressors associated with urbanisation; i.e. higher temperature, increased pollution, and stronger fragmentation. Following the same categorization, Brans et al. (2018) report a clear signal of UHI in the study region, with ponds in urban areas being on average ca. 3.5°C warmer during summer season compared to ponds in rural areas. This UHI had a stong impact on growing season length, with significantly longer seasons in urban than in rural ponds (Brans et al. 2018).

We suggest water bodies in urban areas to be more polluted by contaminants when compared to the more natural rural areas, yet evidence for such an argument requires an ambitious year-long sampling campaign, which is often not feasible due to logistic reasons (as was the case for the present study). Considering rural areas as pristine, natural habitats, it is quite straightforward to expect higher contamination in urban areas, especially given the frequent residential use of pesticides in cities (Budd et al. 2007; Weston et al. 2009), which can end up in urban water bodies due to increased runoff in cities (e.g. Jiang et al. 2012). For the present study, we made sure that the selected rural ponds are not adjacent to agricultural land, given that pesticide application in cropland may cause nearby ponds to be contaminated. Distance to agricultural land and agricultural land coverage surrounding ponds has been associated with pesticide tolerance levels of natural populations (Coors et al. 2009; Cothran et al. 2013). None of our rural ponds were in the immediate vicinity of agricultural land (min. distance 110 m). In chapter 2, we argue in detail that no elevated pesticide tolerance in the studied rural ponds due to agriculture is expected.

In agreement with findings from other regions (discussed above), urbanisation was positively correlated with habitat fragmentation in the study region: aquatic habitats situated in urban areas had higher mean nearest-neigbour distance compared to habitats in rural areas (Merckx et al. in press).

### Traits under investigation

#### *(i) Life history and fitness traits*

In the larval stage, I mainly focused on growth rate (rate of body mass gain) and development time (time from egg hatching to adult emergence). These traits are strongly linked to fitness in damselflies, as they determine the condition (e.g. body mass) in which the individual emerges as an adult. Growth rate and development time (also referred to as 'age at emergence') are sensitive both to temperature and contaminant exposure (e.g. Debecker et al. 2017). Further, these traits are especially relevant response variables for time stress in damselflies (e.g. Mikolajewski et al. 2015), considering the indirect effect of UHIs on growing season length (discussed above). At metamorphosis I measured the mass at emergence. Mass at emergence and development time have been suggested as key traits mediating the effects of larval conditions on adult performance and fitness (Rowe and Ludwig 1991; Abrams et al. 1996; Stoks and Cordoba 2012). During the adult stage, I measured age at maturation (time from emergence to the first mating), lifespan, and lifetime mating success (total number of matings acquired).

### *(ii) Behavioural traits*

I measured activity, boldness, and exploration behaviours, traits commonly used in personality studies (Réale et al. 2007), during the larval stage. Changes in these behaviours may have crucial consequences for fitness (Sih 2013; Wong and Candolin 2015), biotic interactions (Belgrad and Griffen 2016; Santostefano et al. 2016) and even community dynamics (Moran et al. 2017; Start and Gilbert 2017). In chapter 2, I calculated activity as the number of position changes in a given time, whereas in chapter 3, I estimated activity as distance travelled within a given time (after optimizing an automated video tracking tool). Activity values using the two measures are highly correlated (in odonate larvae: Johansson 2000). I measured boldness, defined as the reaction to a risky situation (Réale et al. 2007), either as a change in activity in the presence of predator cues from the baseline conditions (Chapter 2), or as latency time to move after a simulated predator attack (Chapter 3). Finally, to measure exploration (defined as an individuals' reaction to a novel situation; Réale et al. 2007), I quantified activity in a non-familiar arena. I also measured food intake (number of *Artemia* prey consumed in a given time), given its close link to growth rate and personality traits (Biro and Stamps 2008). In chapter 3, I assessed activity and boldness repeatedly to estimate the consistency (i.e. repeatability) of these behaviours; a prerequisite for personality and behavioural syndrome studies (Réale et al. 2007).

### *(iii) Dispersal-related traits*

In chapter 6, I measured flight performance, given its close link to dispersal ability (Baguette et al. 2012). I quantified two parameters: flight endurance, measured as the duration of a flight bout, and flight speed, the speed of the initial flight bout (as in Gyulavári et al. 2014, 2017; Therry et al. 2014b; Tüzün et al. in press). In flying insects, altered flight performance is often driven by changes in flight-related physiological (fat content and flight muscle mass; Therry et al. 2014b; Gyulavári et al. 2014; Tüzün et al. in press) and morphological traits (wing size and shape; e.g. Arambourou et al. 2017; Berwaerts et al. 2002). Therefore, I also measured the fat and muscle contents of adult damselflies, and quantified both size (centroid size) and shape-related (relative warps) wing parameters.

## Statistical analyses

To test for the effects of urbanisation, or a specific treatment, on traits under investigation, I used linear mixed-effect models (LMMs). For non-normally distributed response variables, such as the binomial mating success or Poisson-distributed behavioural traits, I used generalized linear mixed-effect models (GLMMs), with the appropriate error structure. As I worked with replicated populations per urbanisation level, I included population (nested within a given urbanisation level) as a random factor to all models, as this accounts for potential variation attributed to differences between populations, as opposed to urbanisation itself. I used type III sum-of-squares, with dummy coding (i.e. "treatment contrasts" in R-language) for non-ordered factors. Overdispersion for binomial models were controlled for by including an individual-level random factor (Harrison 2014).

When I aimed to study the covariation patterns between traits, as well as to disentangle direct and indirect effects of certain treatments, I applied the path analysis approach. This method is based on structural equation modeling (SEM, Grace 2006), including only observed variables. I specifically applied the generalized multilevel path analysis (also called piecewise SEM; Lefcheck et al. 2016), a state-of-the-art approach that allows for random factors and non-normal distributions (Shipley 2009; Lefcheck et al. 2016).

**Research aims and outline**

The overall aim of my PhD project was to gain insight into the effects of urbanisation on ecological and evolutionary processes. I explicitly focused on three stressors associated with urbanisations: warming, pesticide exposure, and habitat fragmentation. Importantly, I investigated how these urban stressors, both alone and in combination, influence ecological and evolutionary processes across life stages. Finally, I studied how effects of urbanisation on traits may alter sexual selection patterns.

Focusing explicitly on the three urban stressors discussed in detail above, I tested for two mechanisms that would facilitate damselfly populations to colonize and inhabit urban habitats: local adaptation and spatial sorting. Dispersal from the source rural habitats to urban habitats is possibly tightly mediated by spatial sorting, whereby only individuals with the best dispersal capacity can reach the highly fragmented and isolated ponds in urban areas. Signals of spatial sorting would therefore be visible in adult phenotypes, as the dispersive stage of damselflies is the adult phase. Once colonized urban areas, damselflies are faced with additional novel selection pressures, e.g. increased contamination and temperature. I expect adaptive evolutionary responses to these stressors especially in larval traits (the larval stage covers the majority of the damselfly life cycle and especially this stage cannot escape these stressors). These two dynamics, spatial sorting and local adaptation, do not necessarily operate independently from each other: assuming carry-over effects from the larval-to-adult stage, adaptive responses to larval stressors may alter adult traits that have already been shaped by spatial sorting. Hence, spatial sorting and local adaptation can jointly shape the phenotype of the damselfly. A conceptual scheme of these two mechanisms is depicted in Figure 1.

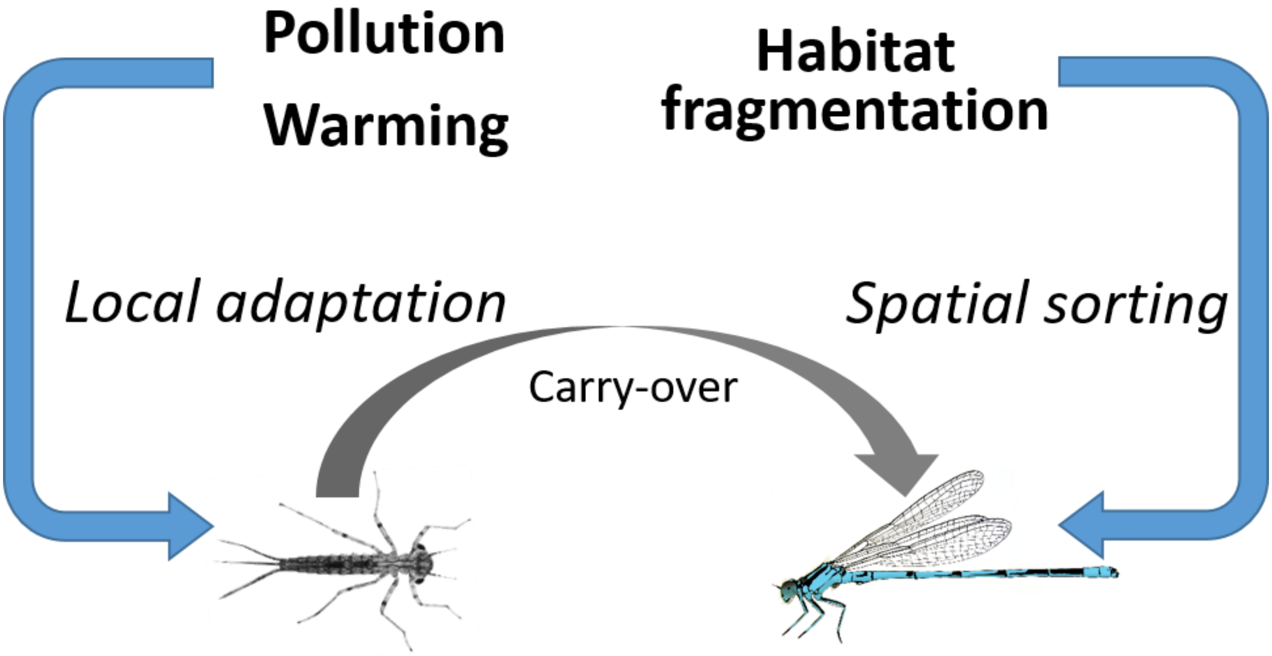

**Figure 1.** A conceptual scheme, depicting the potential urban-related selection pressures acting on damselflies. Pollution and warming are expected to drive local adaptation, primarily acting on the larval stage. Habitat fragmentation is expected to shape adult traits via spatial sorting (see main text for details). The grey arrow represents the potential carry-over effects, connecting the two life stages. Illustrations of the larva and adult damselfly are by Viktor Nilsson-Örtman.

For all but one study presented in this thesis (chapters 1-5), I applied the common garden rearing approach, using eggs collected from urban and rural populations. A potential differentiation in trait expressions between urban and rural populations detected under common garden settings can, to a large extent, exclude environmental factors as the driving force. Hence, responses detected under common garden experiments can be interpreted as genetic adaptations (Kawecki and Ebert 2004), yet with potential influences from maternal effects. This approach is commonly applied, especially for multi-stressor studies (e.g. climate change and contaminants: Kimberly and Salice 2015). In the first two chapters, I carried out the experiments under laboratory settings, while in chapters 4 and 5, I conducted outdoor experiments to enhance realism. As an exception, animals used in chapter 3 were raised in outdoor conditions (as in chapters 4 and 5), yet tested under standardized indoor conditions. To ensure sufficient genetic variation, I used eggs from at least 10 females per population. In chapter 6, I studied sexual selection in natural urban and rural populations and therefore used field-caught adults to infer potential urbanisation-driven changes in flight performance and the underlying flight-related traits. To also investigate the sexual selection on these traits, I collected both unmated and mated males; a common approach to detect sexual selection (e.g. Blanckenhorn et al. 1999; Gosden and Svensson 2008, Gyulavári et al 2014, 2017). A scheme of the thesis chapters where the tested stressors and traits responses are indicated is given in Figure 2.

In chapter 1, I applied the powerful thermal performance curve (TPC) approach to compare whether the relationship between larval growth rate and rearing temperature differs between rural and urban populations. To detect signals of the growth-mortality trade-off (e.g. Hong and Shurin 2015), I also measured larval survival. Given the higher temperatures encountered in urban ponds, I predicted higher performance at higher temperatures (i.e. a horizontal shift in TPC) in urban compared to rural populations (Lynch and Gabriel 1987; Gilchrist 1995). On the other hand, since higher seasonal temperatures can extend the growing season, hence relax time stress, I alternatively expected countergradient variation where the more time-stressed individuals grow faster at each temperature (i.e. a vertical shift in TPC; e.g. Conover et al. 2009; Conover and Schultz 1995).

In chapters 2 and 3, I tackled the question whether urban and rural damselfly larvae differ in their behavioural responses when exposed to pesticides. For this, I measured activity, boldness (both chapters), food intake and exploration behaviours (chapter 2) before and after exposure to pesticide. While focusing on mean levels of behaviours in chapter 2, I explicitly studied personality (consistent individual variation) and behavioural syndromes (consistent behavioural correlations) in chapter 3. I also measured growth rate, as it may correlate with personality traits (Biro and Stamps 2008). Given the well-known interactive effect of pesticide and temperature (Noyes et al. 2009), I additionally crossed the pesticide treatment with a temperature treatment (chapter 2).

In chapters 4 and 5, I studied whether the urbanisation level of individuals determines the way effects of stressors experienced during the larval stage carry over to the adult stage, and influence reproductive success. In addition to manipulating time constraints by using the natural variation in reproductive phenology (early- vs late-hatched larvae), I also applied a pesticide during the larval stage as an urban-related stressor. This longitudinal one-year rearing experiment consisted of an outdoor microcosm part for the larval stage, and a large outdoor insectary part for the adult stage. I measured several key life history traits during the larval stage, at metamorphosis, and during the adult stage. Due to the strongly sex-dependent nature of the responses, I investigated responses of females (chapter 4) and males (chapter 5) in separate chapters.

In the final chapter, I used field-collected adult male damselflies to test for urbanisation-driven differentiation in dispersal-related traits by measuring flight performance and flight-related traits. I further tested whether a potential differentiation in flight performance influences sexual selection regimes.

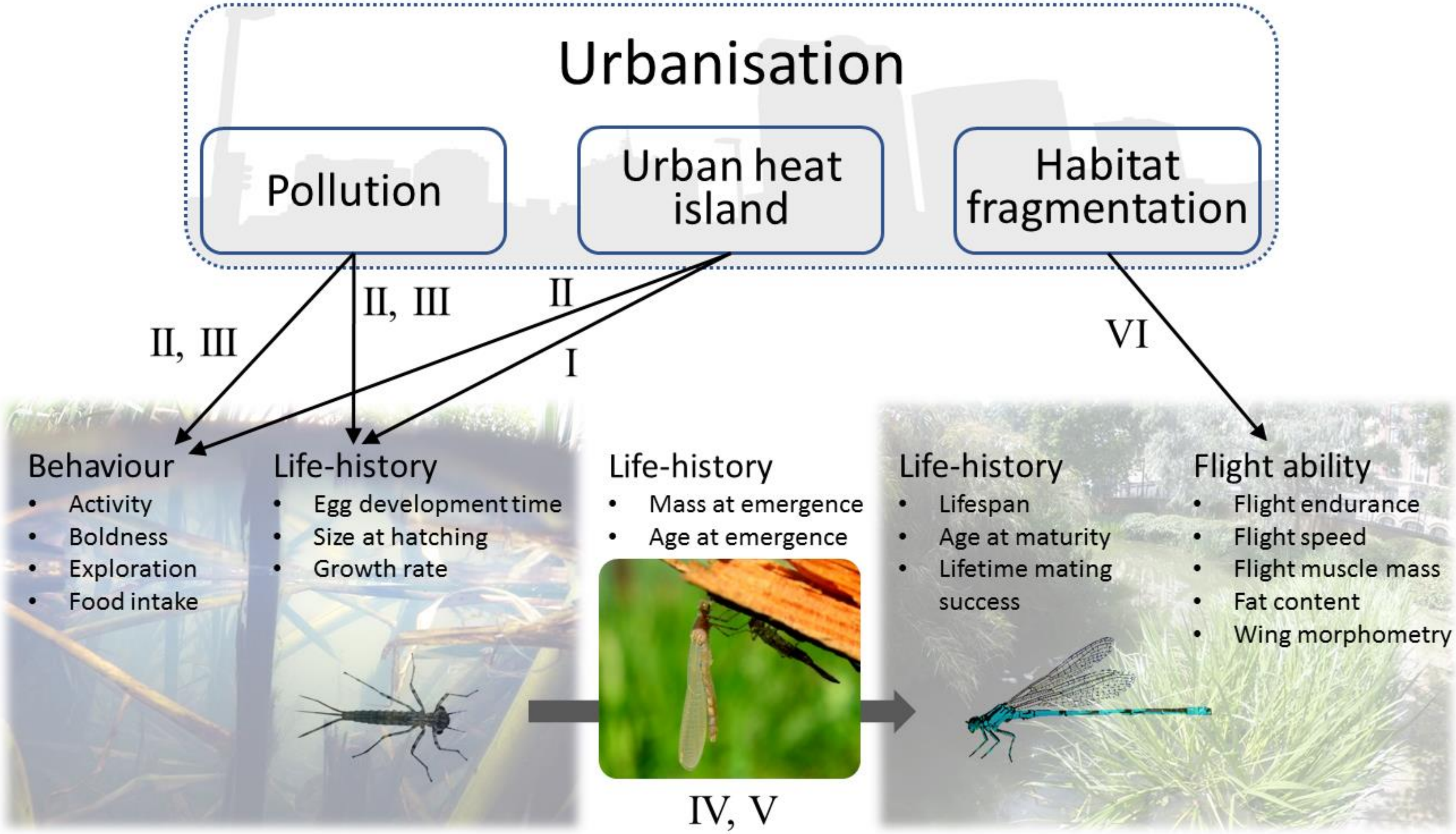

**Figure 2.** Schematic overview of the thesis project. Effects of three urbanisation-related stressors (pollution, urban heat island and habitat fragmentation) were tested either at the aquatic larval stage (left panel), during metamorphosis (central panel), or at the terrestrial adult stage (right panel) of the damselfly *Coenagrion puella*. The arrow in the central panel represents the transition of life stages, as well as illustrates carry-over effects of the stressors. Roman numerals next to arrows indicate the chapter(s) in which the stressors were tested. Listed are the traits that were tested at each life stage. Illustrations of the larva and adult *Coenagrion puella* are by Viktor Nilsson-Örtman.

## CHAPTER 1

# Microgeographic differentiation in thermal performance curves between rural and urban populations of an aquatic insect

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## Abstract

The rapidly increasing rate of urbanisation has a major impact on the ecology and evolution of species. While increased temperatures are a key aspect of urbanisation ("urban heat islands"), we have very limited knowledge whether this generates differentiation in thermal responses between rural and urban populations. In a common garden experiment, we compared the thermal performance curves (TPCs) for growth rate and mortality in larvae of the damselfly *Coenagrion puella* from three urban and three rural populations. TPCs for growth rate shifted vertically, consistent with the faster-slower theoretical model whereby the cold-adapted rural larvae grew faster than the warm-adapted urban larvae across temperatures. In line with costs of rapid growth, rural larvae showed lower survival than urban larvae across temperatures. The relatively lower temperatures, hence expected shorter growing seasons in rural populations compared to the populations in the urban heat islands likely impose stronger time constraints to reach a certain developmental stage before winter, thereby selecting for faster growth rates. In addition, higher predation rates at higher temperature may have contributed to the growth rate differences between urban and rural ponds. A faster-slower differentiation in TPCs may be a widespread pattern along the urbanisation gradient. The observed microgeographic differentiation in TPCs supports the view that urbanisation may drive life history evolution. Moreover, because of the urban heat island effect, urban environments have the potential to aid in developing predictions on the impact of climate change on rural populations.

## Introduction

Urbanisation is rapidly increasing worldwide (Seto et al. 2012), and the differences in environmental conditions between urban and rural areas have a major impact on the ecology and evolution of species (Alberti 2015; Parris 2016; Alberti et al. 2017a,b). A key environmental difference is the considerably higher mean temperature in urban areas ("urban heat islands"; Gaston et al. 2010), leading to extended growing seasons (Yang et al. 2013; Somers et al. 2013; Zipper et al. 2016) and causing shifts in phenology (Neil and Wu 2006; Townroe and Callaghan 2014). Yet, surprisingly few studies looked at trait differentiation in response to urban heat islands, and we lack evidence whether these differences are genetic (reviewed in Chown and Duffy 2015; Diamond et al. 2015; but see Brans et al. 2017a,b).

Considering that the temperature difference between urban and rural areas fall frequently within the range of the expected temperature increases by 2100 under IPCC (2013) scenarios, adaptation to urban environments can inform on the impact of climate change on organisms (Chown and Duffy 2015; Youngstead et al. 2015; 2017).

Thermal performance curves (TPCs), the continuous reaction norms of organismal performance traits (e.g. growth rate) in response to temperature, are useful tools for the study of differentiation in thermal responses (Stinchcombe et al. 2012; Sinclair et al. 2016). The spatial differentiation of TPCs to compensate differences in local temperatures may take three not mutually exclusive adaptive patterns (Yamahira and Conover 2002; Angilletta 2009): (1) The TPCs may be shifted horizontally ("hotter-colder" model), with the thermal optimum being higher in warm-adapted genotypes than in cold-adapted genotypes as predicted by theory (Lynch and Gabriel 1987; Gilchrist 1995). (2) The TPCs may be shifted vertically ("faster-slower" model), with cold-adapted genotypes having a higher performance than warm-adapted genotypes at all temperatures (for continuous thermal spatial gradients referred to as countergradient variation) (Conover and Schultz 1995; Conover et al. 2009). Under countergradient variation, genetic and environmental effects oppose each other to produce similar performance in the natural populations along a thermal gradient. (3) Based on the suggested trade-off between maximal performance and thermal breadth, the TPCs may differ in their width ("generalist-specialist" model), with the performance breadth being narrower in genotypes with higher maximum performance than in genotypes with lower maximum performance. It is theoretically predicted that genotypes adapted to high compared to low temperature variations would have broader TPCs (Lynch and Gabriel 1987; Gilchrist 1995), yet studies have provided mixed empirical support (Angilletta 2009). While "faster-slower" differentiation is not predicted by optimality models of thermal evolution (Lynch and Gabriel 1987; Gilchrist 1995), it is often documented for growth rate where it is believed to be driven by time constraints as the increased growth rates in colder environments compensate for the shorter growing season (Conover et al. 2009).

We here focused on geographic differentiation in TPCs for growth rate, a commonly used performance trait (Angilletta 2009), between replicated urban and rural populations of an aquatic insect using a common garden rearing experiment with a range of temperatures.

While urbanisation gradients correspond to temperature gradients (Parris 2016; for the here studied urbanisation gradient: De Ridder et al. 2015; Brans et al. 2017a), we focused on the extreme urban and rural populations of the gradient. Note that this approach is a relevant and powerful setting to test for the type of spatial differentiation in TPCs (horizontal vs. vertical shift). To better interpret the TPC pattern for growth rate and possible costs, we also reconstructed TPCs for three other key life-history traits: larval survival, egg development time and hatchling body size. As study species we chose the damselfly *Coenagrion puella*, which is very abundant in both rural and urban areas in Europe (Goertzen and Suhling 2013). We studied rural and urban population in Flanders. At this spatial scale, populations of *C. puella* experience high gene flow (Johansson et al. 2013), hence our study entails microgeographic differentiation sensu Richardson et al. (2014). As ectothermic invertebrates, damselfly larvae are especially sensitive to temperature and have been documented to show thermal adaptation at a macrogeographic (e.g. latitudinal) scale (e.g. Shama et al. 2011; Nilsson-Örtman et al. 2012; De Block et al 2013). Very few studies focused on effects of urbanisation in damselflies (Tüzün et al. 2015; Villalobos-Jiménez et al. 2016; Villalobos-Jiménez and Hassall 2017) and these did not consider differentiation in TPCs.

## Materials and methods

### *Study species and populations*

Adult *C. puella* reproduce in early summer and eggs hatch ca. 3 weeks later. Larval development takes ca. 10 months (Lowe et al. 2009). This species is univoltine in central Europe (Corbet et al. 2006). We studied three rural populations (Bierbeek, Bornem and Houwaart) and three urban populations (Leuven, Mechelen and Oudenaarde), all situated within a 45 km radius in Flanders, Belgium. All ponds are shallow water bodies with abundant aquatic vegetation. The selection of urban and rural ponds was carried out following a two-step procedure using Geographic Information System (GIS). First, three urban and three rural 3 x 3 km plots were selected based on the percentage of built-up area: >15% for urban plots and <3% for rural plots. Second, we selected in each plot a pond in a subplot of 200 x 200 m with the same urbanisation level. This ensured that both the direct environment (subplot) and the broader surroundings (plot) reflected the same urbanisation level. This sampling design was applied in several recent studies where effects of urbanisation on organisms are investigated (Tüzün et al. 2015; Kaiser et al. 2016; Brans et al. 2017a; Piano et al. 2017). We collected eggs from 10 mated females from each of 6 ponds in July 2013.

*Experimental setup*

We set up a full factorial common garden experiment with five rearing temperatures crossed with two levels of urbanisation (urban and rural, each represented by three populations). Animals were reared from the egg stage at one of five constant temperatures: 16, 20, 24, 28 and 30 °C. Mean water temperatures experienced by the study species are typically in the range 16-20 °C during the early and intermediate periods of development in summer and early fall (Nilsson-Örtman et al. 2013). Eggs and larvae may experience higher water temperatures during summer (R. Stoks, unpublished data). Simulations with the lake model Flake (Lake Model Flake 2016) confirmed the occurrence of the here used extreme temperatures in the study region during summer. Although we did not use the more realistic daily fluctuating temperature regime, the ranking of trait values among mean temperature treatments have been shown to be largely unaffected by fluctuating vs. constant temperature regimes (e.g. Fischer et al. 2011). We assigned 30 individuals (3 larvae per female) per population to each temperature (total of 900 individuals).

Eggs and larvae were kept in dechlorinated tap water and placed in incubators at a photoperiod of 14:10 h light/dark (reflecting the late summer – early fall photoperiod at the study region) at one of the five rearing temperatures. Larvae were reared individually in 200 ml plastic cups and were fed *Artemia* nauplii five days a week (mean ± SE: 212 ± 67 nauplii per feeding portion, $n$ = 12 feeding portions), corresponding to high food levels.

To estimate growth rate, we measured the head width of each larva on days 0 (newly hatched), 30 and 50 using a digital camera attached to a binocular microscope. Head width is an often used measure to estimate size and growth in damselfly larvae (for an example in the study species: Mikolajewski et al. 2005). The repeated measurements of head width allowed testing for ontogenetic changes in the thermal growth curves (Nilsson-Örtman et al. 2013). As the thermal sensitivity of growth rates in the study species strongly depends on the ontogenetic stage (Van Doorslaer and Stoks 2005; Nilsson-Örtman et al. 2013), we calculated growth rate separately for the periods between days 0 – 30 and days 30 – 50 (from here on referred to as first and second period, respectively). This period spans the important growth period of *C. puella* larvae during late summer and early fall. For this, we used the formula [ln(final head size) – ln(initial head size)]/number of days. In animals experiencing seasonal time constraints, such as the here studied damselfly *C. puella* (Lowe et al. 2009; Mikolajewski et al. 2015), growth rate is a relevant performance trait. Moreover, rapid larval growth is important to reach a size advantage in cannibalistic interactions in this study species (Rolff 1999), especially immediately following hatching when the densities are high.

We calculated larval survival as the ratio of larvae that survived up to day 50. Details on egg size, egg development time and larval size at hatching are reported and discussed in Appendix S1. The experiment was terminated at day 50, when larvae were old enough to weigh them without causing damage (see Appendix S2 for details**)**.

### *Statistical analyses*

Unless stated otherwise, all analyses were conducted with R version 3.2.2 for Windows (R Development Core Team 2015). We used the package 'lme4' (Bates et al. 2015) for mixed-effects models, and the package 'car' to compute Wald $\chi^2$ statistic and *p*-values for fixed effects (Fox and Weisberg 2011). Significant interactions were further analyzed by comparing least-square means using Tukey post hoc analysis.

To assess the effects of urbanisation level and rearing temperature on growth rate and survival, we used separate (generalized) linear mixed-effects models. We tested for the effects of urbanisation level (urban and rural) and temperature (both linear and quadratic term) by including these terms, and their interactions, as fixed effects. As an exception, growth period (first and second period) was included as an additional fixed effect to the growth rate model. We included hatchling size as a covariate to the growth rate model. The following random effects were added where appropriate: population, nested within urbanisation level (accounting that animals from the same pond are not independent replicates, thereby avoiding pseudoreplication), female identity of the offspring (accounting for among-brood variation), individual identity of larvae (there were two growth rate estimates, one per period, for each larva; i.e. repeated-measures design). We provide a detailed summary of the model structures in Appendix S3.

In order to decompose the variation in TPCs into contributions of “hotter-colder”, “faster-slower” and “generalist-specialist” models, we used the Template Mode of Variation (TMV; Izem and Kingsolver 2005) using the code by Izem and Kingsolver (2005) implemented for Matlab (v.8.6.0). This method uses a polynomial function for the decomposition, where each direction of variation is represented by changes in curve-specific parameters (i.e. height, width and optimum temperature). Based on the detected urban-rural differentiation in TPCs using linear mixed-effects models (see Results), we applied this method on growth rates during the second period.

## Results

### *Larval growth rates*

Larvae with smaller hatchling size had higher growth rates ($\chi^2 = 20.9$, d.f. = 1, $P < 0.001$). Both the linear (Temperature × Growth period: $\chi^2 = 225.2$, d.f. = 1, $P < 0.001$) and the quadratic effect of temperature on growth rate (Temperature$^2$ × Growth period: $\chi^2 = 142.2$, d.f. = 1, $P < 0.001$) differed between the two growth periods. In addition, growth rates were ca. 2.5 times higher during the first period (0.041 day$^{-1}$) compared to the second period (0.017 day$^{-1}$) ($\chi^2 = 8176$, d.f. = 1, $P < 0.001$). During the first period, increasing temperatures resulted in an increase in growth rate up to 28 °C where a plateau was reached (Fig. 1A). During the second period, the TPC had a concave downward shape with decreasing growth rates at temperatures above 24 °C (Fig. 1B).

Growth rates were higher in rural larvae than in urban larvae, but only in the second period (Urbanisation level × Growth period: $\chi^2 = 6.72$, d.f. = 1, $P = 0.01$; contrast test, first period: $P = 0.97$; second period: $P = 0.02$, Fig. 1B). The effect of temperature on growth rate was similar for rural and urban larvae, and this was consistent across growth periods (all interactions with temperature $P > 0.4$).

The TMV analysis explained 64.65 % of the total variation in TPCs. In line with the results from the linear mixed-effects model, the TMV analysis of the growth rate during the second period revealed that the vertical shift ("faster-slower" model) explained the majority of the variation (58.82 %) in TPCs of urban and rural larvae, whereas the horizontal shift of the TPCs ("hotter-colder" model) and the "generalist-specialist" model accounted only for 3.76 % and 2.07 % of the variation, respectively. Additional outputs of the TMV analysis are reported in Appendix S4.

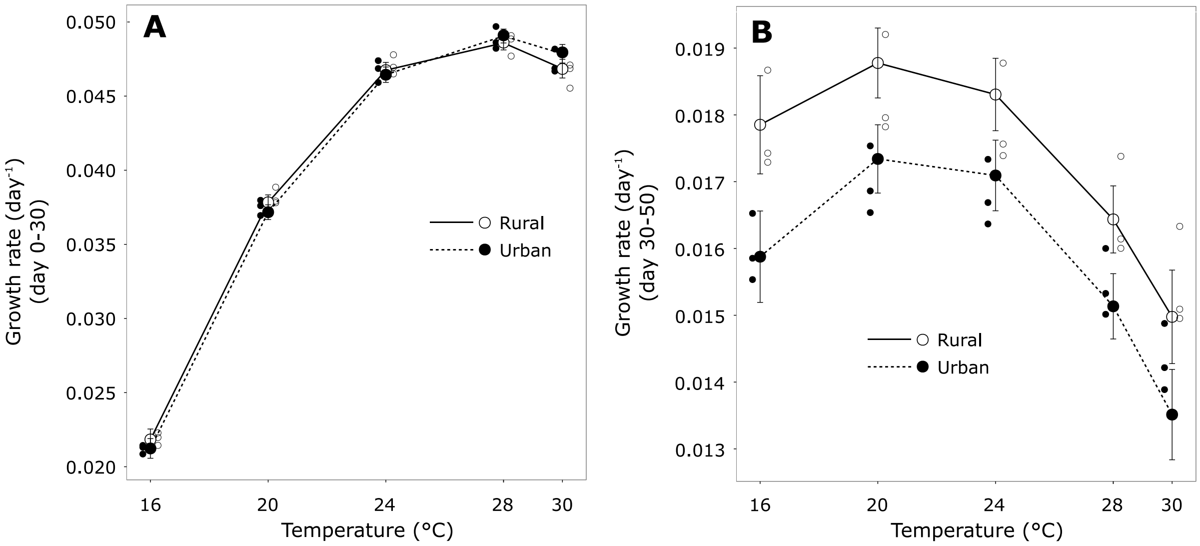

**Figure 1.** Thermal performance curves for larval growth rate between (A) days 0 – 30 and (B) days 30 – 50 of urban and rural populations of the damselfly *Coenagrion puella*. Note the different scales of the vertical axes. Small circles represent means of individual populations of a given urbanisation level. Least-square means ± 1 SE are shown.

### *Larval survival*

Larval survival was ca. 90 % at intermediate temperatures, and decreased to ca. 60 % both at the lowest (16 °C) and the highest (30 °C) temperature, resulting in an inverted U-shaped TPC (Fig. 2); a pattern supported by the highly significant quadratic effect of temperature ($\chi^2$ = 122.3, d.f. = 1, $P < 0.001$). Urban larvae had higher survival compared to rural larvae ($\chi^2$ = 5.43, d.f. = 1, $P = 0.02$), and this was consistent along the temperature range (Urbanisation level × Temperature: $\chi^2$ = 0.38, d.f. = 1, $P = 0.53$; Urbanisation level × Temperature$^2$: $\chi^2$ = 0.41, d.f. = 1, $P = 0.52$).

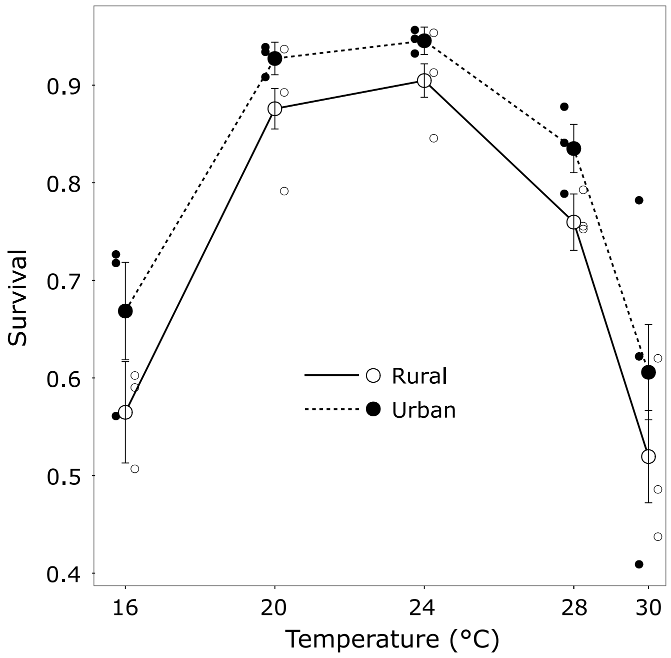

**Figure 2.** Thermal performance curves for larval survival of urban and rural populations of the damselfly *Coenagrion puella*. Small circles represent means of individual populations of a given urbanisation level. Least-square means ± 1 SE are shown.

## Discussion

We found solid support for microgeographic differentiation of the TPCs for growth rate between urban and rural populations. Average surface temperatures during summer have been reported to be between 1.4 – 4.5 °C, and in extreme cases up to 8 °C, warmer in urban compared to rural areas in Flanders (De Ridder et al. 2015). In line with this, a recent study reported a 4.03 °C difference in mean summer maximum water temperature in a subset of two urban and two rural ponds used in this study (Brans et al. 2017a). Despite the higher water temperatures in urban ponds, the TPCs for growth rate were not shifted horizontally towards higher optima in urban damselfly populations. Furthermore, although daily temperature fluctuations are more pronounced in urban than in rural ponds in the study region (Brans et al. 2018), there was no differentiation in the width of the TPCs. Instead, TPCs where shifted vertically, with rural larvae having higher growth rates across rearing temperatures during the second period. The observation that the vertical shift in growth TPCs was consistent across the three sampled populations per urbanisation level suggests this pattern to be general (see Appendix S5 and S6 for more detail). While the here chosen design of replicated populations studied at the extreme ends of the urbanisation gradient is powerful to detect microgeographic shifts in TPCs, our setup does not allow to infer that these shifts occur gradually across the urbanisation gradient. As we will argue that time constraints associated with changes in temperature likely are driving the vertical shift in TPCs, and given that temperatures gradually change along urbanisation gradients (Parris 2016; for the here studied urbanisation gradient: De Ridder et al. 2015; Brans et al. 2017a), gradual changes in TPCs are expected. This will, however, require further studies also including populations sampled at intermediate positions along the urbanisation gradient.

There was a pronounced ontogenetic shift in the TPC for growth rate. While during the first period, growth rates were faster and the TPC had a steep slope and a high thermal optimum, there was a shift in the second period toward lower growth rates and a downward concave-shaped TPC with a lower thermal optimum. Such ontogenetic shifts in TPC for growth have been associated with the decreasing temperatures larvae are exposed to during early and intermediate growth periods, and have been reported before in *Coenagrion* damselfly species, including the study species *C. puella* (Van Doorslaer and Stoks 2005; Nilsson-Örtman et al. 2013). The high thermal optimum in the first period (28 °C) is not often reached in the study ponds. This fits the pattern that thermal optima are typically higher than modal environmental temperatures (Angilletta et al. 2010). This has been explained by the typically asymmetric shape of TPCs resulting in drastically lower performance above the thermal optimum than below the thermal optimum (Deutsch et al. 2008). Even if the optimal temperatures are met infrequently, it may still be beneficial to optimize growth at higher temperatures as even short exposures to higher temperatures may allow significant gains (Kingsolver 2000). Selection for high growth rates and reaching a larger size may be stronger in early larval stages because of the initial high larval densities (leading to increased cannibalism; Hopper et al. 1996), and higher temperatures (leading to increased competition and intraguild predation; Brown et al. 2004). This, together with the identified trade-off between early and late growth rates in *Coenagrion* damselfly larvae (Nilsson-Örtman et al. 2013) may have caused the higher growth rates in the first compared to the second period. Alternatively, the lower growth rates in the second period could be an artefact driven by size-dependent growth rate (Tammaru and Esperk 2007), where the lower growth rates of the larvae during the second period were due to their on average larger size. This scenario, however, is unlikely to drive the observed pattern as we controlled for the larval size (by including either initial size or time-varying sizes as covariate; see Appendix S2) when analyzing growth rate. Note that given size is fixed per developmental stage, this also corrects for any differences in developmental stage between temperature treatments. Although we followed the growth rate TPCs only until day 50, this captures the major part of the pre-winter growth period. Moreover, for the study species, the shape of the TPCs between days 30 – 50 reflects the TPCs until the end of the pre-winter growing season (based on Van Doorslaer and Stoks 2005; Nilsson-Örtman et al. 2015).

A key finding of our study was the vertical shift in TPC for growth rate during the second period with higher growth rates in rural larvae compared to urban larvae. This vertical shift in TPCs is consistent with a pattern of countergradient variation (Conover and Schultz 1995; Conover et al. 2009). We did not find any evidence for the theoretically predicted higher thermal optima ("hotter-colder" model, Lynch and Gabriel 1987; Gilchrist 1995) of urban damselfly larvae that would suggest thermal adaptation. The majority of evidence for thermal adaptation comes from interspecific studies (e.g. Frazier et al. 2006; Angilletta et al. 2010), whereas intraspecific studies usually favour the "faster-slower" model (e.g. Yamahira and Conover 2002; Izem and Kingsolver 2005; Richter-Boix et al. 2015). It has been suggested that thermal adaptation ("hotter-colder" model) requires radical changes in the genetic structure (e.g. mutations that would allow for an increased growth via horizontal shifts in thermokinetics of enzyme function), which requires time of the magnitude similar to what is needed for the divergence of species (Yamahira and Conover 2002; Yamahira et al. 2007). Furthermore, in spite of the higher daily temperature fluctuations in urban ponds (Brans et al. 2018), urban populations did not have wider TPCs ("generalist-specialist" model; Lynch and Gabriel 1987; Gilchrist 1995). This matches the general pattern that thermal specialisation is only encountered infrequently (Angilletta 2009; but see Latimer et al. 2011; Richter-Boix et al. 2015).

The majority of studies reporting vertical shifts in TPCs for growth rates show this pattern across large geographic gradients with little gene flow among populations (Conover et al. 2009), whereas only a handful of studies demonstrated this pattern at a microgeographic scale where gene flow can be high (but see Blanckenhorn 1991; Skelly 2004; Richter-Boix et al. 2010, 2015). Also in the here studied *C. puella*, gene flow is high at the studied scale in Flanders (as indicated by the very low $F_{ST}$-values of ca. 0.02 for this study species, see Figure 2 in Johansson et al. 2013). This indicates that selection for higher growth rates is especially strong to counteract the homogenizing force of gene flow, thereby supporting accumulating evidence that local adaptation may occur at small spatial scales in the presence of gene flow (reviewed in Richardson et al. 2014).

Given that we obtained the TPC differentiation in a common garden experiment suggests that the pattern reflects genetic adaptation, rather than environmental differences. Although we used first generation animals, maternal effects seem unlikely to have played a major role because rural and urban populations did not differ in egg size and hatchling size (Appendix S1), while differences in egg size are the most common way how maternal effects are transferred (Mousseau and Fox 1998). More importantly, larval growth rates did not differ between rural and urban populations during the first period. It seems unlikely that any non-genetic effects transferred by the mother would have caused differences in growth rate between population types that would not be present during the first growth period. Indeed, if anything, maternal effects tend to decay throughout ontogeny (e.g. Lindholm et al. 2006). This fits the pattern based on quantitative genetic rearing experiments that maternal effects on growth rate are absent or small in damselfly larvae (Shama et al. 2011; Sniegula et al. 2016). Nevertheless, we cannot fully exclude mechanisms acting via maternal effects such as transgenerational plasticity (e.g. Richter-Boix et al. 2014).

The observation that the vertical shift in TPCs only was apparent in the second period may be explained by the stronger time constraints; the major selective force underlying a vertical shift in TPCs for growth rates (Conover et al. 2009). Indeed, during the second period, temperatures encountered in the field are lower while larvae face stronger time constraints to reach a certain developmental stage before the onset of winter. In support of a stronger selection to accelerate growth rates in rural populations, growing seasons are reported to be considerably shorter in rural than in urban areas (Yang et al. 2013, Somers et al. 2013; for the study region: Brans et al. 2018). Higher growth rates under time constraints have been theoretically predicted (Abrams et al. 1996) and empirically shown in *Coenagrion* damselfly larvae, including the study species (Mikolajewski et al. 2015). However, systematic differences between urban and rural ponds other than temperature might have contributed to the differentiation in TPCs. Notably, differences in predation pressure have been identified as a key factor driving the shift in TPCs for growth rate (Richter-Boix et al. 2013). Predators of damselfly larvae did, however, not differ in densities between the here studied urban and rural ponds (see Appendix S7). In agreement with our finding, aquatic macro-invertebrate community structures are not driven by urbanisation in the study region (Caroline Souffreau, personal communication), and a recent study reported no difference in species richness of aquatic macro-invertebrates from urban and nonurban ponds in the United Kingdom (Hill et al. 2017). Nevertheless, even when predator densities did not differ between urban and rural ponds, predators may impose stronger selection for reduced growth rates in the urban ponds because of the typical higher predation rates at warmer temperatures (e.g. De Block et al. 2013; Sentis et al. 2015). Larvae of the study species indeed reduce growth rates in the presence of predators (Mikolajewski et al. 2005). The higher temperatures in the urban populations may therefore be driving the lower growth rates in urban populations both by causing lower time constraints and by generating higher predation rates. Furthermore, differences in pollution levels between pond types might have contributed to the observed shift in TPCs. Indeed, tadpoles exposed to a herbicide had on average upwards shifted ("faster-slower" differentiation) and narrower TPCs ("generalist-specialist" differentiation) for swimming speed (Katzenberger et al. 2014). Yet, the expected higher contamination levels in urban waterbodies (e.g. Gilliom 2007), together with the absence of a generalist-specialist trade-off in our study makes this scenario unlikely.

Despite the widespread evidence of urban heat islands (Gaston et al. 2010), surprisingly few studies documented differentiation in TPCs between rural and urban populations, and as these all directly measured traits on field collected animals it is unknown to what extent these differentiations are genetic rather than environmentally driven (reviewed in Chown and Duffy 2015). In the only study considering continuous TPCs, which is essential to discriminate between the models (Richter-Boix et al. 2015), two different patterns of thermal differentiation were observed when rearing four species of soil fungi directly isolated from one urban population and from one rural population (McLean et al. 2005). Two fungus species followed the "hotter-colder" model, with isolates from the urban population growing faster at 26°C, but slower at 18°C compared to isolates from the rural population. In the two other fungus species, urban isolates grew as fast or faster at all temperatures than the rural isolates (“faster-slower” model). This is the opposite vertical shift in TPCs of that we here documented. The authors suggested this is because high temperatures in urban areas inhibit fungal growth, resulting in restricted growing seasons for urban populations (McLean et al. 2005), while the higher temperatures in the urban damselfly populations, instead, were beneficial and increased the length of the growing season. In contrast, two studies report higher heat tolerance in urban compared to rural populations of field-collected leafcutter ants (Angilletta et al. 2007) and common garden-reared acorn ants (Diamond et al. 2017), yet none of them tested differential thermal responses in life history. Finally, Brans et al. (2017b) have shown genetic adaptation to urbanisation in terms of a higher heat tolerance in urban populations of the water flea *Daphnia magna*.

Studies on vertical shifts in TPCs typically assume life history trade-offs where the faster growing genotypes pay costs that preclude them from occupying the entire thermal gradient (Conover and Schultz 1995; Conover et al. 2009). Costs of rapid growth may be manifold (Dmitriew 2011), and in damselfly larvae include decreased investment in energy storage (Stoks et al. 2006), reduced immune function (De Block and Stoks 2008b), and increased oxidative stress (De Block and Stoks 2008a). These costs may explain the overall lower survival in rural compared to urban populations. Similarly, Hong and Shurin (2015) showed that northern populations of the tidepool copepod *Tigriopus californicus* grew faster than southern populations, yet suffered a reduced survival. However, we cannot exclude the possibility that adaptations to urbanisation-related stressors (e.g. pollution) might have resulted in an overall higher resilience of urban populations, reflected in their higher survival.

Urbanisation is a major driver of microevolutionary change (Alberti 2015; Alberti et al. 2017a). Urban environments have the potential to provide unique information and aid in developing predictions on the impact of climate change on organisms (Chown and Duffy 2015, Youngstead et al. 2015; 2017), and the use of cities as natural experiments has recently been promoted. Our finding that individuals from urban and rural populations consistently differed in growth rate and survival is relevant for predicting climate change impact, as the summer temperature difference between urban and rural ponds falls within the range of the expected temperature increases by 2100 under several IPCC (2013) scenarios. The urban populations can therefore be used as proxies to understand and predict the impact of global warming in rural populations under gradual evolution (Stoks et al. 2014). Aside from contributing to the limited evidence for vertical shifts in TPCs at a microgeographic scale (Skelly 2004; Richter-Boix et al. 2015), this study is the first using replicated populations in a common garden experiment from the egg stage to report evidence for urbanisation-associated countergradient variation in a key performance trait.

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## Appendix S1 – TPCs for egg development time and size at hatching

We determined egg size (prior to the temperature treatments) by measuring the length of five random eggs per clutch using a digital camera attached to a binocular microscope. To determine size at hatching, we measured the head width of each larva on day 0 (i.e. directly after hatching) using a digital camera attached to a binocular microscope. We defined egg development time as the duration between oviposition and egg hatching.

To assess the effects of urbanisation level and rearing temperature on egg size, hatchling size, and egg development rate, we used separate (generalized) linear mixed-effects models. We tested for the effects of urbanisation level (urban and rural) and temperature (both linear and quadratic term) on various response variables by including these terms, and their interactions, as fixed effects. As an exception, temperature was not included in the egg size model (egg sizes are fixed and do not change with temperature). In addition, models for egg development time and hatchling body size included egg size as a covariate. Finally, the following random effects were added where appropriate: population, nested within urbanisation level (accounting that animals from the same pond are not independent replicates, thereby avoiding pseudoreplication), and female identity of the offspring (accounting for among-brood variation). We provide a detailed summary of the model structures in Appendix S4. We used the R package 'lme4' (Bates et al. 2015) for mixed-effects models, and the package 'car' to compute Wald $\chi^2$ statistic and *p*-values for fixed effects (Fox and Weisberg 2011).

Egg size did not differ between urbanisation levels ($\chi^2$ = 0.42, d.f. = 1, $P$ = 0.52). Egg development time decreased with increasing egg size ($\chi^2$ = 4.04, d.f. = 1, $P$ = 0.04). Increasing temperatures resulted in a four times faster egg development, going from ca. 29 days at 16 °C to ca. 7 days at 30 ° C (linear effect temperature: $\chi^2$ = 28.72, d.f. = 1, $P$ < 0.001) (Fig. S1A). The quadratic effect of temperature was significant as well ($\chi^2$ = 7.65, d.f. = 1, $P$ = 0.01), indicating the temperature effect was strongest at the lower temperatures (Fig. S1A). The quadratic TPC for egg development time did not differ between urban and rural eggs (Temperature × Urbanisation level: $\chi^2$ = 0.001, d.f. = 1, $P$ = 0.98; Temperature$^2$ × Urbanisation level: $\chi^2$ = 0.003, d.f. = 1, $P$ = 0.95), neither was there a fixed difference in development time between urban and rural eggs (χ2 = 1.83, d.f. = 1, $P$ = 0.18).

Hatchling body size (measured as larval head width at hatching) was affected by temperature, with intermediate temperatures resulting in a greater body size (linear effect of temperature: $\chi^2 = 61.11$, d.f. = 1, $P < 0.001$, quadratic effect of temperature: $\chi^2 = 71.53$, d.f. = 1, $P < 0.001$, Fig. S1B). The quadratic TPC for hatchling size did not differ between urban and rural larvae (Temperature × Urbanisation level: $\chi^2 = 0.18$, d.f. = 1, $P = 0.67$; Temperature² × Urbanisation level: $\chi^2 = 0.18$, d.f. = 1, $P = 0.67$), neither was there a fixed difference in initial size between urban and rural larvae ($\chi^2 = 2.60$, d.f. = 1, $P = 0.11$). Egg size did not have a significant effect on hatchling body size ($\chi^2 = 1.82$, d.f. = 1, $P = 0.18$).

While egg development time and hatchling size showed clear thermal responses, this was not associated with thermal adaptation. As observed in many other taxa (Gillooly et al. 2002; including aquatic insects: Pritchard et al. 1996; Gillooly and Dodson 2000) increasing temperatures shortened the egg development time. This has been explained by the metabolic theory of ecology: due to the thermodynamics of enzyme-catalysed reactions, biological rates are predicted to increase with temperature (Gillooly et al. 2001; Brown et al. 2004). This may also have contributed to the decline in hatchling size at higher temperatures, which was previously observed in the study species by Van Doorslaer and Stoks (2005). This apparent trade-off between egg development times and hatchling size has been described before in various taxa, including insect species (e.g. hawkmoth: Potter et al. 2011; a tropical butterfly: Geister et al. 2009). These studies have argued that the mechanisms underlying the so-called temperature-size rule, where high developmental temperatures result in small adult size (Atkinson 1994), apply to embryonic development as well. The biophysical model by van der Have and De Jong (1996) suggests that high temperatures accelerate cell division, while the increase in cellular growth does not catch up with the cell division rate. This may be a possible explanation for larger hatchlings at lower temperatures.

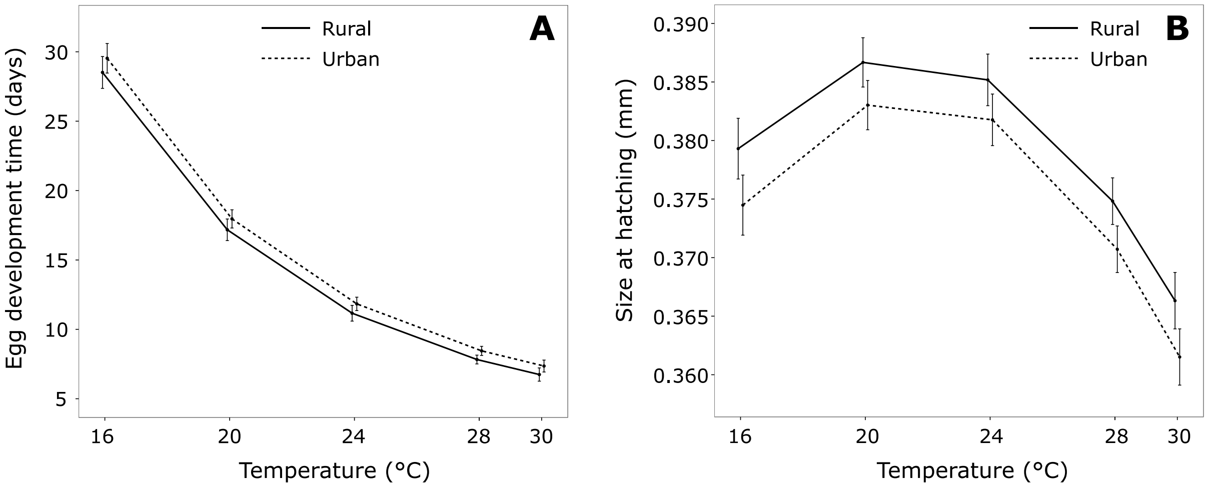

**Figure S1**. Thermal performance curves for (A) egg development time and (B) size at hatching of urban and rural populations of the damselfly *Coenagrion puella*. Egg development time was calculated as the time between oviposition and hatching. Size at hatching was measured as head width of newly emerged larvae. Least-square means ± 1 SE are shown.

## Appendix S2 – Complementary growth rate analyses

We could not directly determine increases in body mass as larvae are too fragile to handle during early stages. To assess whether the head width measurements also reflected patterns in body mass, we quantified the correlation of head width and wet body mass at the end of the experiment (day 50). At that moment, all larvae were weighed to the nearest 0.01 mg after gently blotting them dry with tissue paper before weighing to ensure that no water remained on the larva. Head width and wet mass measured at day 50 were highly correlated ($r = 0.94$, $P < 0.001$).

Given that comparisons of growth patterns may be confounded with size differences in case of size dependent growth rates, we took special precautions based on Tammaru and Esperk (2007) and Nicieza and Álvarez (2009). When analysing growth rate, we included larval size at hatching as a covariate to the model (see main text). In addition to this, we also ran a related model with a time-varying size-related covariate: size at hatching for growth during the first period, and the size at the start of the second period for growth during the second period. Moreover, we ran a separate model with a repeated-measures design using the subsequent sizes of a given larva (at days 0, 30 and 50) as repeated response variable (instead of the two growth rates) (Nicieza and Álvarez 2009). Population (nested within urbanisation level) and female identity of the offspring was included as random effects to all models. We used the R package ‘lme4’ for constructing (generalized) linear mixed models (Bates et al. 2015) and the ‘car’ package to compute Wald $\chi^2$ statistic and *p*-values for fixed effects (Fox and Weisberg 2011).

The finding of the period-dependent urbanisation effect on growth rate during the second period (see Results in main text) did not change qualitatively when including size as time-varying covariate (Urbanisation level × Growth period: $\chi^2 = 8.96$, d.f. = 1, $P = 0.003$), or when using size as response variable in a repeated-measures model (Urbanisation level × Growth period: $\chi^2 = 18.8$, d.f. = 2, $P < 0.001$) indicating this pattern is no artefact of size differences between periods.

## Appendix S3 – Structure of mixed-effect models

**Table S1.** Model structures used for testing effects of urbanisation level and temperature on various response variables. Error structure, fixed effects, covariates, and random effects used in the models are shown separately for each response variable. Note that in all cases except for egg size, both the linear and quadratic term of temperature was included as fixed effects. Urb = urbanisation level, Temp = temperature, Period = growth period.

| Response variable | Error structure (function) | Fixed effects | Covariate | Random effects |
|---|---|---|---|---|
| Growth rate | Normal<br>(identity link) | Urb, Temp, Period,<br>Urb × Temp,<br>Urb × Period, Temp × Period,<br>Urb × Temp × Period | Hatchling size | Population + Female ID + Individual ID |
| Survival | Binomial<br>(logit-link) | Urb, Temp,<br>Urb × Temp | - | Population + Female ID |
| Egg size | Normal<br>(identity link) | Urb | - | Population + Female ID |
| Hatchling size | Normal<br>(identity link) | Urb, Temp,<br>Urb × Temp | Egg size | Population + Female ID |
| Egg development rate | Poisson<br>(log-link) | Urb, Temp,<br>Urb × Temp | Egg size | Population + Female ID |

## Appendix S4 – TMV parameters

**Table S2.** Parameters obtained from the TMV method. w = width (dimensionless); m ($T_{opt}$) = optimal temperature; h = height ($day^{-1}$); $Z_{max}$ = maximum performance ($day^{-1}$). We refer to Izem and Kingsolver (2005) for detailed explanations of the parameters.

| Urbanisation level | w | m ($T_{opt}$ °C) | h | $Z_{max}$ |
|---|---|---|---|---|
| Rural | 0.9876 | 20.138 | 0.0009 | 0.0189 |
| Urban | 1.0113 | 21.636 | -0.0009 | 0.0167 |

## Appendix S5 – Testing for the consistency of populations within a given urbanisation level

Aside from including population (nested within urbanisation level) as a random effect to each model (see Statistical analyses in main text), we constructed additional models to test whether populations from the same urbanisation level were consistent in their response to the temperature gradient. For this, we ran separate models for urban and rural individuals where we included the interaction term of population and temperature (both linear and quadratic term). A lack of significant interactions would indicate the consistency of populations within a given urbanisation level. We did this for growth rate during the second period (i.e. day 30-50) and larval survival, as for these response variables a significant urbanisation level effect was detected (see Results in main text). These models also included larval size at hatching as a covariate, and female identity of the offspring as a random effect. We used the R package 'lme4' for constructing (generalized) linear mixed models (Bates et al. 2015) and the 'car' package to compute Wald $\chi^2$ statistic and *p*-values for fixed effects (Fox and Weisberg 2011).

In terms of growth rate, rural populations did not differ in their response to the temperature gradient (Population × Temperature: $\chi^2 = 1.35$, d.f. = 2, $P = 0.51$; Population × Temperature²: $\chi^2 = 1.64$, d.f. = 2, $P = 0.44$), and neither did urban populations (Population × Temperature: $\chi^2 = 4.94$, d.f. = 2, $P = 0.08$; Population × Temperature²: $\chi^2 = 4.47$, d.f. = 2, $P = 0.11$).

Analysis of larval survival revealed that urban populations did not differ in their response to the temperature gradient (Population × Temperature: $\chi^2 = 5.3$, d.f. = 2, $P = 0.07$; Population × Temperature²: $\chi^2 = 5.6$, d.f. = 2, $P = 0.06$). We found a significant interaction between rural populations and the linear ($\chi^2 = 14.2$, d.f. = 2, $P < 0.001$) and quadratic term of temperature ($\chi^2 = 15.8$, d.f. = 2, $P < 0.001$), indicating that populations from rural ponds differed in their response to the temperature gradient. Visual inspection of Figure 3 (main text) suggested this was because of the populations showing considerable variation in survival at 30 °C. At the set of other temperatures, there were no significant interactions between population and the linear or quadratic term of temperature (rural populations: Population × Temperature: $\chi^2 = 0.94$, d.f. = 2, $P = 0.62$; Population × Temperature$^2$: $\chi^2 = 0.92$, d.f. = 2, $P = 0.63$; urban populations: Population × Temperature: $\chi^2 = 1.99$, d.f. = 2, $P = 0.37$; Population × Temperature$^2$: $\chi^2 = 1.97$, d.f. = 2, $P = 0.37$), indicating that for this temperature range populations from the same urbanisation level were consistent in their response to the temperature gradient.

## Appendix S6 – Assessing the influence of random effects

To assess the influence of the random effects population and family, we compared the marginal $R^2$, the proportion of variance explained by the fixed factors only, and the conditional $R^2$, the proportion of variance explained by both the fixed and random factors (see e.g. Peay et al. 2015). Marginal and conditional $R^2$ values were calculated based on Nakagawa and Schielzeth (2013) for mixed-effects models. We used the R package 'piecewiseSEM' to obtain marginal and conditional $R^2$ values (Lefcheck, 2015).

Fixed effects alone explained 88.76 % of the variance in the growth model (marginal $R^2$), whereas the variance explained by both the fixed and random effects was 88.82 % (conditional $R^2$); indicating that the inclusion of the random effects (i.e. population nested within urbanisation level and identity of the larvae nested within the female identity) did not increase the explanatory power.

As in the growth rate model, the inclusion of the random effects (i.e. population nested within urbanisation level and identity of the female) resulted in a negligible increase in explanatory power: fixed effects alone explained 21.41 % of the variance in the survival model (marginal $R^2$), whereas the variance explained by both the fixed and random effects was 21.69 % (conditional $R^2$). This also indicates that any differences in the response of the populations of the same urbanisation level were minor.

## Appendix S7– Predator communities

Given that differences in predator densities may contribute to differences in growth rate (e.g. Laurila et al. 2008), we estimated the densities of predators feeding on damselfly larvae. To this end, we conducted dipnet sweeps (3 m haul distance) at five different locations in each pond using a D-frame dipnet (0.5 mm mesh size) on 7 October 2016. The five locations per pond were selected based on the presence of submerged aquatic vegetation where damselfly larvae and their predators frequently occur. Predator densities were presented as the sum of the five samplings per pond. Differences in predator community composition between urban and rural ponds were visualized using non-metric multidimensional scaling. We compared total predator densities of urban and rural ponds with a t-test.

Although predator density differed between ponds (Table S3), this was not consistent among populations from the same urbanisation level (*t* = 0.987, d.f. = 2.044, *P* = 0.426). Similarly, ponds had distinct predator assemblages, yet they did not cluster based on their urbanisation level (Fig. S2).

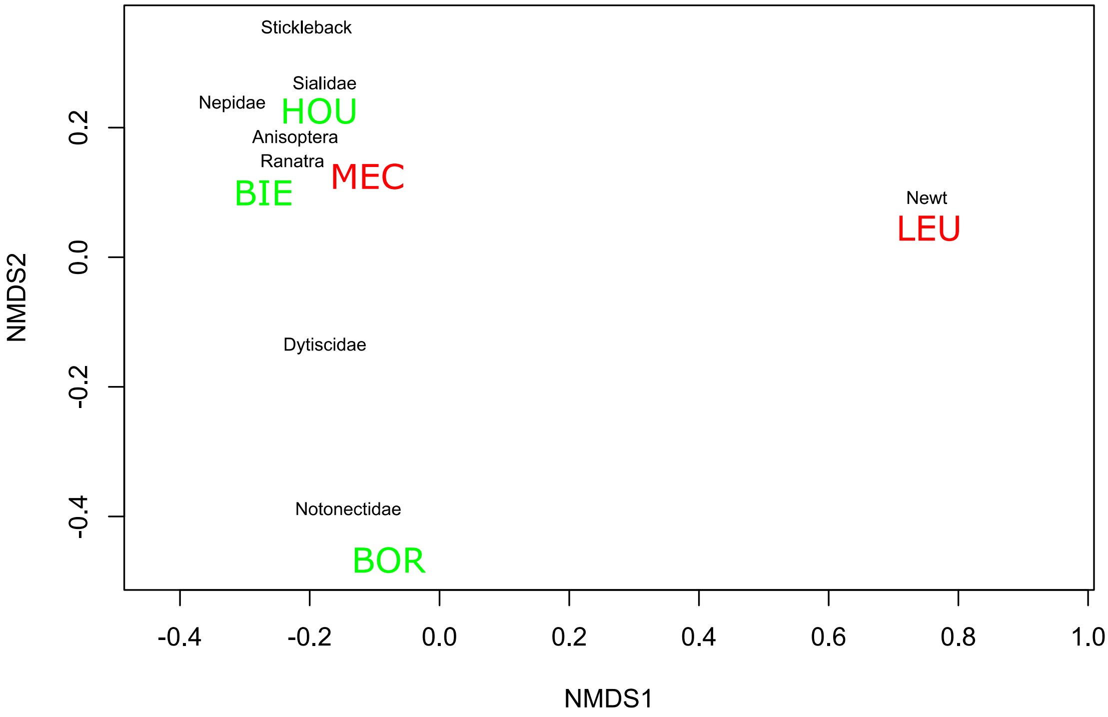

**Figure S2**. Non-metric multidimensional scaling of damselfly predator communities among the study ponds (Bie: Bierbeek, Bor: Bornem, Hou: Houwaart, Leu: Leuven, Mec: Mechelen).

**Table S3**. Community and densities of damselfly predators at each of the six study ponds. Numbers per pond are the sum of the five samples per pond. Taxa were categorized as damselfly predators based on McPeek 1990; Corbet 1999; Siepielski et al. 2010.

| | **Rural populations** | | | **Urban populations** | | |
|---|---|---|---|---|---|---|
| | Bierbeek | Bornem | Houwaart | Leuven | Mechelen | Oudenaarde |
| **Anisoptera** | 8 | 0 | 1 | 0 | 4 | 0 |
| ***Ranatra linearis*** | 6 | 0 | 0 | 0 | 1 | 0 |
| **Notonectidae** | 21 | 56 | 20 | 0 | 11 | 0 |
| **Nepidae** | 2 | 0 | 1 | 0 | 0 | 0 |
| **Dytiscidae** | 16 | 7 | 8 | 0 | 44 | 0 |
| **Sialidae** | 0 | 0 | 24 | 0 | 27 | 0 |
| **Newts** | 0 | 0 | 0 | 5 | 0 | 0 |
| **Sticklebacks** | 0 | 0 | 6 | 0 | 0 | 0 |
| **Total** | 53 | 63 | 60 | 5 | 87 | 0 |

# CHAPTER 2

## Urbanisation shapes behavioural responses to a pesticide

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## Abstract

The degree of urbanisation is rapidly increasing worldwide. Due to anthropogenic impact, urban populations are exposed to higher levels of contaminants and higher temperatures. Despite this, urbanisation is a largely overlooked spatial component in ecotoxicology. We tested in a common garden rearing experiment whether replicated urban and rural populations of the damselfly *Coenagrion puella* differ in their vulnerability to sublethal levels of a widespread pesticide, chlorpyrifos, in terms of ecologically relevant behaviours (exploration behaviour, activity, boldness and food intake), and to what extent these patterns are affected by temperature (20 and 24°C). Except boldness, all behaviours were affected by previous pesticide exposure. While the pesticide did not affect exploration behaviour at 20°C, it was associated with increased exploration at 24°C, which may reflect an increased toxicity of chlorpyrifos at higher temperatures. Importantly, rural and urban larvae showed consistently different, sometimes even opposite behavioural responses to pesticide exposure. When exposed to the pesticide, rural larvae decreased activity and food intake at both temperatures; urban larvae instead increased activity at both temperatures and only reduced food intake at the high temperature. This suggests that urban larvae were less affected by the pesticide, which would be consistent with a scenario of local adaptation to higher contaminant levels. Our results highlight that urbanisation may be an important factor to arrive at a spatially explicit ecological risk assessment, and may be an ignored reason why studies on the same species may generate widely different vulnerabilities to pesticides.

## Introduction

Despite increasing attention for spatially explicit ecological risk assessment (Van den Brink 2008; Clements et al. 2012) and the rapidly increasing urbanisation levels worldwide (UN 2014), urbanisation is a largely overlooked spatial component in ecotoxicology. Urbanisation is regarded as a major threat to ecological communities (McKinney 2008), as ecosystems in urban areas suffer from a range of anthropogenic pressures, including substantial accumulation of contaminants in urban surface water (Gilliom 2007). It is, however, unknown to what extent this generates local adaptation where urban populations are better adjusted to deal with contaminants. Such patterns may be expected as many studies indicated patterns of local adaptation where populations experiencing higher contaminant levels evolved a lower vulnerability to these contaminants (reviewed in Medina et al. 2007; but see e.g. Crespi 2000; Spitzer 2006).

Due to regulatory programs, most freshwater organisms in developed countries are only rarely facing lethal levels of pesticides; yet there is increasing concern that also sublethal pesticide levels may have profound effects (Köhler and Triebskorn 2013), eventually leading to biodiversity loss (Beketov et al. 2013). One important type of response variables that may mediate negative effects of sublethal pesticide doses are changes in ecologically relevant behaviours (Weis et al. 2001; Dell'Omo 2002; Zala and Penn 2004; Desneux et al. 2007). Contaminants at sublethal doses have been shown to affect behaviours such as activity (e.g. Relyea and Mills 2001), boldness (e.g. Brodin et al. 2013), and food intake (e.g. Zubrod et al. 2014) in a wide range of taxa. Maladaptive behavioural changes that have a direct effect on trophic relations, such as predator-prey interactions, can have major implications for both the prey (e.g. reduced survival, Janssens and Stoks 2012) and for the predator (e.g. reduced growth, Campero et al. 2007), which may eventually propagate through the food web (reviewed in Rohr et al. 2006). Given that behavioural responses to contaminants may have a genetic base (e.g. Breckels and Neff 2010), it may be expected that locally adapted urban and rural populations consistently differ in their behavioural responses to pesticides.

Besides contamination, higher temperatures are another key abiotic stressor associated with urbanisation, creating the so-called urban heat islands (Oke 1973; Arnfield 2003). Temperatures in urban areas have been recorded to be up to 8°C warmer compared to rural areas (e.g. Rosenzweig et al. 2009; Maiheu et al. 2013). These two stressors usually do not act independently, as many contaminants are known to be more toxic at higher temperatures, which is of considerable concern in a warming world (Noyes et al. 2009; Moe et al. 2013). Urban ecosystems have therefore been proposed as suitable study systems with the potential to provide valuable insights into the effects of global warming in a contaminated world (Grimm et al. 2008b; Youngsteadt et al. 2014). As urban populations are generally exposed to both contamination and temperature increases (Gilliom 2007; George et al. 2007), these populations may develop local adaptations making them less vulnerable to the increased toxicity of pesticides at higher temperatures (for an example along a large-scale temperature gradient, see Dinh Van et al. 2013). Yet, thermal adaptation along temperature gradients may not always mitigate the toxicity of a contaminant at higher temperatures (e.g. Janssens et al. 2014).

In this study, we tested in a common garden rearing experiment from the egg stage whether replicated urban and rural populations of an aquatic insect consistently differ in their vulnerability to sublethal levels of a widespread pesticide in terms of ecologically relevant behaviours, and to what extent these patterns are affected by temperature. As study organisms we used damselfly larvae, important intermediate predators in aquatic food webs (Johnson 1991; Stoks and Córdoba-Aguilar 2012) that are specifically vulnerable to global warming (Hassall and Thompson 2008). We focused on following fitness-related behaviours (Smith and Blumstein 2008): exploration behaviour, activity and food intake (in the presence and absence of predator cues), and boldness. As model pesticide, we chose chlorpyrifos (CPF), one of the most frequently used pesticides worldwide (Eaton et al. 2008). CPF has been shown to alter various behaviours in damselflies; moreover, these effects were often magnified at higher temperatures (Janssens and Stoks 2013b; Dinh Van et al. 2014a, 2014b).

## Materials and methods

### *Study populations and rearing protocol*

We sampled six populations of the damselfly *Coenagrion puella*, one of the most common damselflies in Central Europe (Askew 1988), occupying ponds in urban as well as natural areas (Goertzen and Suhling 2013). We selected three ponds from urban and three from rural areas in Flanders. The selection of the ponds was carried out following a two-step procedure using Geographic Information System (GIS). In a first step, six plots (3 x 3 km) were selected to represent two urbanisation levels defined by the percentage of anthropogenic built-up areas: urban (>15%) and rural (<3%). In a second step, we selected in each plot a pond in a subplot (200 x 200 m) with the same urbanisation level. This ensured that both the direct environment (subplot) and the broader surroundings (plot) reflected the same urbanisation level. All ponds were shallow water bodies with abundant aquatic vegetation, and contained large aeshnid dragonfly larvae as predators. Note that the chosen rural populations were not directly embedded by cropland (distance to nearest cropland: 110 – 500 m), and instead were always near to forest (distance to forest: 0 – 40 m) and surrounded by a high percentage of woodland (35 – 95%). It is therefore unlikely they were affected by agriculture (Declerck et al. 2006).

We collected between 9 and 11 mated females per population during July-August 2013, and transferred them to the laboratory. Females were placed individually in 200 ml plastic cups and provided with wet filter paper for oviposition. For the first 10 days after hatching, larvae were kept in groups at 22°C in order to enhance survival (De Block and Stoks, 2003). Thereafter, larvae were allocated individually to plastic cups (200 ml) with aged tap water, and kept in temperature-controlled water baths (20°C or 24°C) with a constant photoperiod of 14:10 h light/dark. Larvae were daily fed *Artemia* nauplii *ad libitum* (daily food ration: 212 ± 67 nauplii, mean ± SE, *n*=12). We chose these temperatures as 20°C reflects a common summer water temperature in ponds inhabiting the study species in Flanders, while temperatures of 24°C are also encountered, yet much less frequently (Jessie Engelen, unpublished data). The 4°C temperature difference also reflects the predicted 4°C temperature increase by 2100 as predicted under IPCC scenario RCP8.5 (IPCC 2013). Moreover, a recent report shows that in Flanders (e.g. city of Ghent) the temperature in the urban centre is on average 3°C (and up to 8°C) warmer than the bordering countryside (Maiheu et al. 2013), indicating that the 4°C temperature difference used in our study is within the expected temperature difference between urban and rural areas.

### *Experimental setup*

To test for effects of pesticide exposure, temperature and the urbanisation level of the pond of origin on the four behavioural traits (exploration behaviour, activity, food intake and boldness) of the larvae, we set up a full factorial common garden experiment with eight treatment combinations: two pesticide treatments (chlorpyrifos/control), two temperature treatments (20/24°C), and two levels of urbanisation (urban/rural, each represented by three populations). After being randomly assigned to a rearing temperature of 20°C or 24°C, larvae were kept individually throughout the experiment at their designated temperature and also their behaviour was quantified at this temperature. Per female, larvae were randomly distributed across treatment combinations. Due to some mortality during the pre-exposure period, the number of larvae tested at each treatment combination varied between 28 and 50 (total of 303 larvae). Due to logistical constraints, a small number of larvae (*n*=13) could only be tested for exploratory behaviour, and not for activity, food intake and boldness (Table 1).

**Table 1**. Description of the study populations with sample sizes used in the behavioural tests.

| Population | Urbanisation level | No. of females | Number of larvae tested for | |
|---|---|---|---|---|
| | | | Exploration behaviour | Activity, boldness and food intake |
| Mechelen | Urban | 11 | 43 | 40 |
| Oudenaarde | Urban | 9 | 47 | 45 |
| Leuven | Urban | 11 | 81 | 80 |
| Houwaart | Rural | 11 | 44 | 43 |
| Bierbeek | Rural | 9 | 48 | 46 |
| Bornem | Rural | 10 | 40 | 36 |

Once larvae moulted into their final instar (when they were 161±16 days old, mean ± SE, *n*=338), they were randomly assigned to one of the pesticide treatments, consisting of an exposure to pulses of either pesticide or control solution for 15 days. The medium was renewed every three days (static renewal experiment). An ethanol-based chlorpyrifos stock solution with a concentration of 1 mg/ml was used to prepare a second, water-based stock solution of 10 µg/ml (renewed every week). Stock solutions were stored in the dark at 4°C. The latter solution was used to prepare the final working concentration of 2 µg/l chlorpyrifos (nominal concentration), which was used for the pesticide pulses. This concentration was chosen because it modifies antipredator behaviour in coenagrionid damselfly larvae (Dinh Van et al. 2014b), and is within the range of chlorpyrifos concentrations reported in surface waters (Schulz 2004). Pooled samples from ten different vials of the CPF treatment were analysed by the research laboratory Lovap NV (Geel, Belgium) using gas chromatography in combination with mass spectrometry (Thermo-Finnigan Trace DSQ). The initial chlorpyrifos concentration in the experimental cups was 1.24 µg/l. After three days (just before the renewal of the medium), the concentration was measured as 0.83 µg/l and 0.30 µg/l for the 20°C and 24°C temperature treatments, respectively. The control solution consisted of 2 µl/l ethanol to match the ethanol concentration in the chlorpyrifos treatment. We applied the pesticide (and control) solutions as six pulses during 15 days (three times per week). Throughout the exposure period, the larvae were fed daily the same amount of *Artemia* as during the pre-exposure period.

Larvae were weighed to the nearest 0.01 mg before and after the 15-day pesticide exposure period. Each larva was gently blotted dry with tissue paper before weighing to ensure that no water remained on the larva. This allowed us to accurately calculate the growth rate of each individual across the exposure period using the formula ($\ln_{\text{final mass}} - \ln_{\text{initial mass}}$)/15 days. During the exposure period, we checked individuals daily for survival. One day after the pesticide exposure period, we tested the exploration behaviour, activity, food intake and boldness of each larva in the absence of the pesticide.

*Exploration behaviour*

Exploration behaviour of the damselfly larvae was measured by recording activity in a novel environment (Réale et al. 2007). To score exploration behaviour, larvae were placed individually into containers (22 x 11 x 10 cm) filled with 250 ml aged tap water. A grid with 0.5 x 0.5 cm squares taped under the container allowed to locate the position of the larvae. The observation begun after a 10 minute acclimatisation period. We recorded the position of the larvae using the grid coordinates every 10 minutes for 2.5 hours. Exploration was estimated as the number of position changes (defined as the displacement of the centre of the head to another square on the grid), resulting in exploration scores ranging from zero to 15. In order to investigate the frequency of a larva visiting the same square more than once in the same trial (which would not truly represent explorative behaviour), we performed a separate experiment (details below). We found that only very rarely (2.8 ± 2.5% of the observations, mean ± SD) did a larva visit the same square twice, and no larvae visited the same square more than twice during the trials. Therefore, we find it highly unlikely that visiting the same square twice would have influenced our results.

*Activity, food intake and boldness*

Following the exploration test, we measured activity and food intake as ecologically relevant traits. As suggested by Réale et al. (2007), activity was measured in a non-risky and non-novel environment. In addition, we measured the same traits in the presence of predator cues, and used the response to the predator cues as a measure of boldness (Réale et al. 2007).

After the exploration test, larvae were kept in the same container for another 4 hours to minimize the presence of any remaining exploration-related behaviour. To assess whether activity levels were no longer influenced by exploration behaviour at this moment, we performed a separate experiment where we quantified grid movements of individuals ($n = 16$) over a period of time after introduction in the test container (after 30 min, 1 h, 3 h, 6 h and 12 h). We observed that larvae had high activity levels during the first 3 hours after being entered in the container, and then substantially reduced their activity levels after 6 hours (Tukey contrasts: 30 min vs. 6 h, $p = 0.078$; 1 h vs. 6 h, $p < 0.001$; 3 h vs. 6 h, $p < 0.001$), and kept their activity levels stable for the next 6 hours (Tukey contrast: 6 h vs. 12 h, $p = 0.963$). This indicates that the container loses its novelty after 6 hours, and that current activity levels therefore do not reflect exploration behaviour.

The activity test started after adding 4 ml of a standard solution of brine shrimp nauplii (ca. 1600 *Artemia* nauplii) into the container. After 2 minutes, we started monitoring the position of the larvae (as described in the exploration test) once every minute for 8 minutes ('activity without predator cues'). In addition to this behaviour, we recorded walking bouts (defined as bouts of movements where at least one leg changed position) of the larvae. To quantify food intake we measured the amount of nauplii eaten during 8 minutes by direct observation ('food intake without predator cues'). Next, we added 4 ml of predator medium to the container. The predator medium was prepared by homogenizing one *C. puella* larva in 40 ml of water in which a large *Anax imperator* dragonfly larva had eaten one *C. puella* larva. This cocktail of predator cues has been proven to elicit antipredator behaviour in damselfly larvae (Stoks et al. 2003; Janssens and Stoks 2012). Starting after 2 minutes, we measured the same variables as described above for a second observation period of 8 minutes to measure activity and food intake with predator cues. To quantify boldness, we calculated the relative change in grid movements and food intake in the presence of predator cues from the baseline conditions (absence of predator cues). Specifically, we used the formula boldness $= (y_2 - y_1) / y_1$, with $y_1$ indicating the trait value without predator cues and $y_2$ the trait value with predator cues (cf. Hulthén et al. 2014). This resulted in two measures of boldness, one for grid movements and one for food intake. We measured behaviours without predator cues first to avoid any carry-over effects of these cues. We have previously shown in coenagrionid damselfly larvae that no saturation occurs during the second observation period, and that any changes in behaviour between both observation periods are due to the predator cues (Janssens and Stoks 2012).

*Statistical analyses*

We tested for effects of the pesticide treatment, temperature and urbanisation level on the different response variables using a set of linear models. We added population nested within urbanisation level to each model as a random effect. All analyses were performed with the R software version 3.0.2 for Windows (R Development Core Team 2015), using the 'lme4' package for constructing linear mixed models (Bates et al. 2015), and the 'car' package to compute Wald chi-square statistic and *p*-values for fixed effects (Fox and Weisberg 2011).

To test whether survival was influenced by the experimental treatments, we used a generalized linear mixed model (GLMM) with a binomial error distribution and a logit-link function. We analysed growth rate during the pesticide-exposure period using an ANOVA with a normal error structure and the identity link. To determine whether exploration, activity and food intake of damselfly larvae were influenced by the experimental treatments, we used GLMMs with a Poisson error structure and a log-link function. To test whether boldness was affected by the experimental treatments, we used linear mixed models (LMMs) with a normal error distribution and the identity link function. Note that we fitted separate models for the two types of activity (with and without predator cues), the two types of food intake (with and without predator cues) and the two types of boldness (relative change in grid movements and in food intake). Significant interactions were further analysed by comparing least-square means using Tukey posthoc tests. For all models, we included body mass (measured before the behavioural assays on the same day) as a covariate to correct for any potential effects on behaviour (De Block et al. 2013).

We will not report effects on the number of walking bouts due to its strong positive correlation with the number of grid movements both in the absence (Pearson's correlation, $r = 0.66$, $n = 290$, $p < 0.001$) and in the presence (Pearson's correlation, $r = 0.63$, $n = 290$, $p < 0.001$) of predator cues.

## Results

Survival during the pesticide exposure period was overall high (73.7%) and did not differ between urbanisation levels ($\chi^2{}_1 = 0.011$, $p = 0.915$), nor was it affected by the pesticide ($\chi^2{}_1 = 1.150$, $p = 0.284$) (Fig. 1A-B). Larvae raised at 24°C showed a ca. 14% higher mortality than those raised at 20°C ($\chi^2{}_1 = 11.710$, $p < 0.001$). Growth rate did not significantly differ between any of the treatment (pesticide, temperature and urbanisation) groups, nor were their significant interactions (all $p > 0.100$) (Fig. 1C-D).

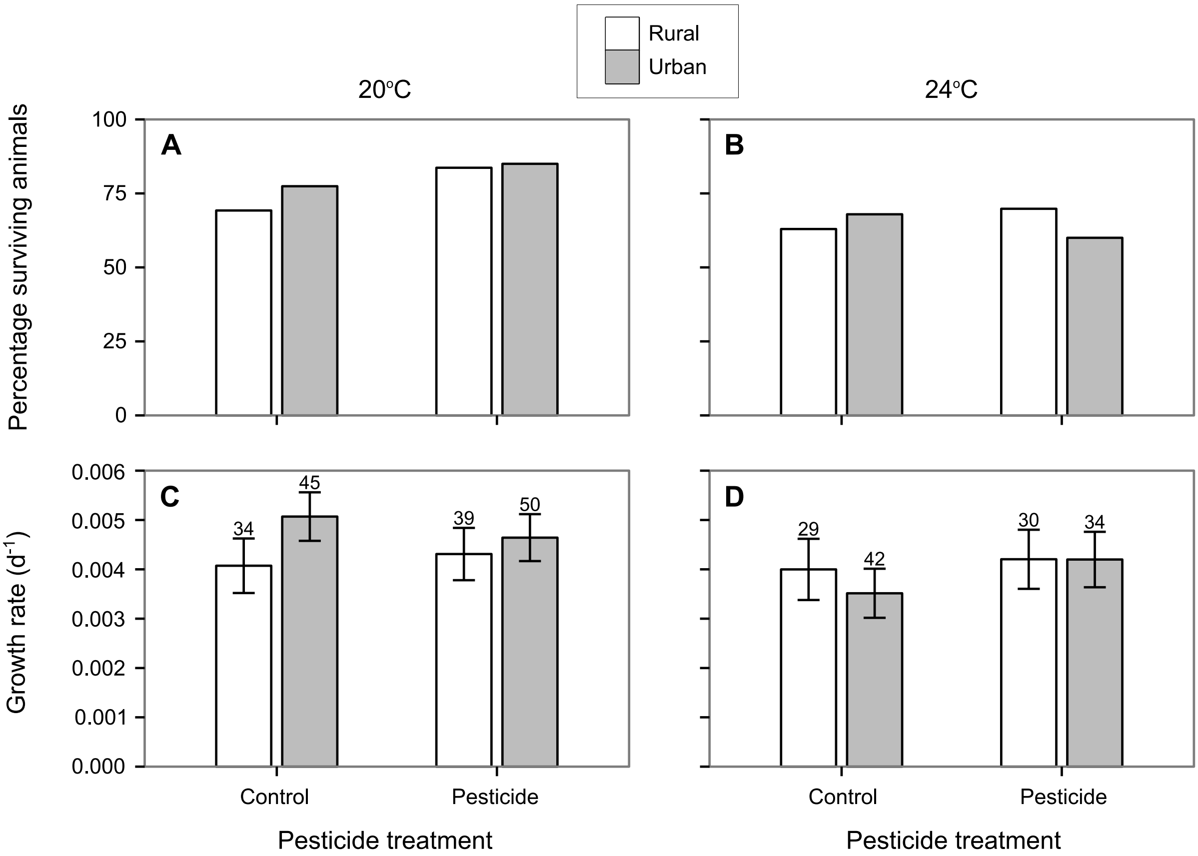

**Figure 1.** Percentage of survival until the behavioural tests (A-B) and mean growth rate (C-D) of *C. puella* larvae in function of pesticide exposure and urbanisation level at 20°C (A and C) and 24°C (B and D). Least-square means ± 1 SE are shown for growth rate. Numbers above the bars represent sample sizes.

*Exploration behaviour*

The effect of previous pesticide exposure on exploration behaviour was dependent on the temperature larvae were raised at (Pesticide × Temperature, Table 2, Fig. 2A-B): pesticide-exposed larvae had significantly higher exploration rates compared to the pesticide-control group for larvae raised at 24°C (Tukey test: $p = 0.033$), whereas larvae raised at 20°C did not change their exploration behaviour when exposed to the pesticide ($p = 0.164$). Exploration behaviour did not differ between urban and rural larvae.

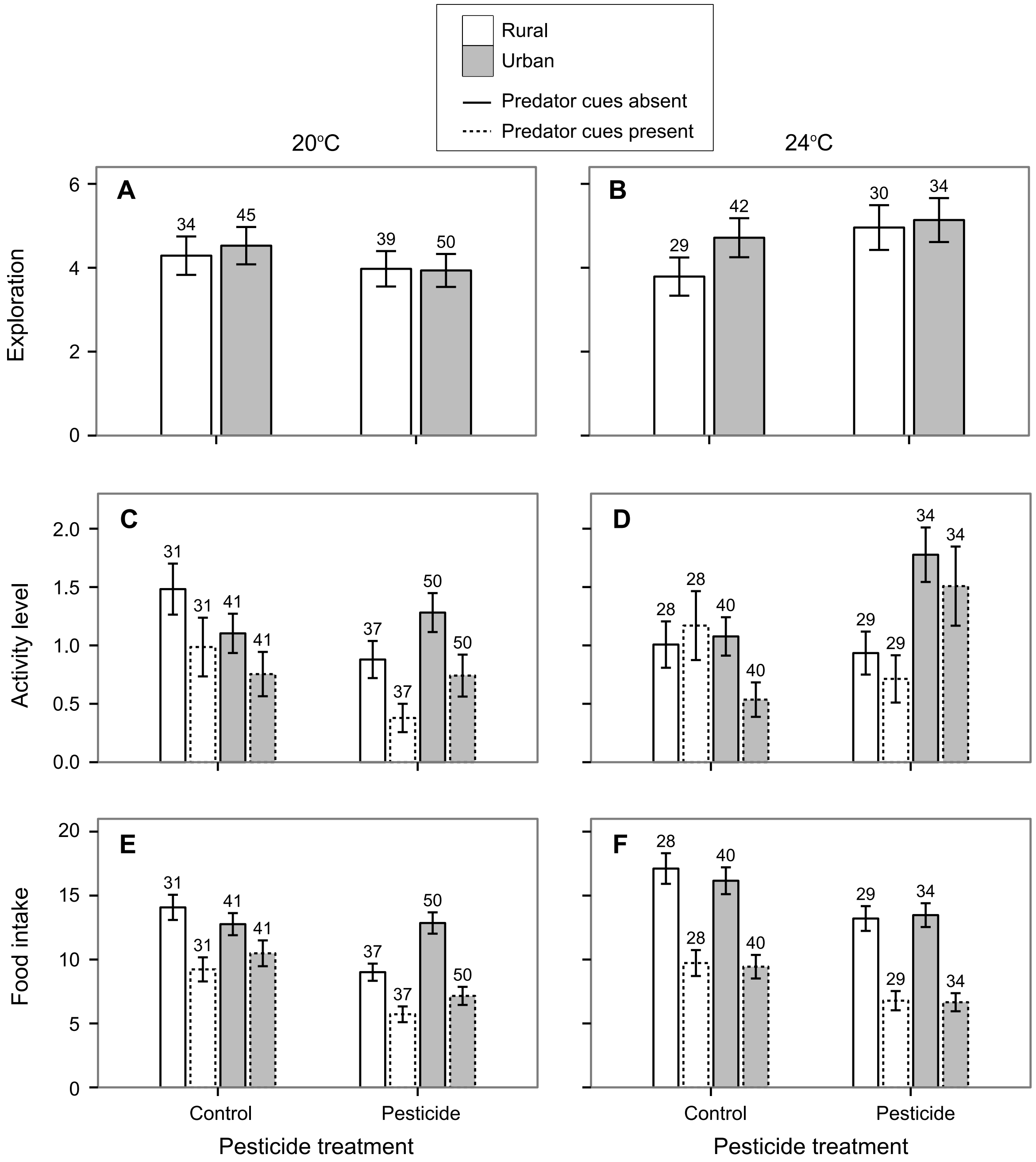

**Figure 2.** Exploration (A-B), activity (C-D) and food intake (E-F) of *C. puella* in function of pesticide exposure, urbanisation level and predator cue exposure at 20°C (A,C,E) and 24°C (B,D,F). Exploration and activity was measured by the number of grid movements, whereas food intake was measured by the number of *Artemia* nauplii eaten. Least-square means ± 1 SE are shown. Numbers above the bars represent sample sizes.

**Table 2.** Results of generalized linear mixed models testing the effects of the pesticide treatment, temperature and the urbanisation level on behaviour. Significant *p*-values are shown in bold. Population (nested in urbanisation level) was included as a random factor.

| Effect | Exploration | | Activity (Without predator cues) | | Activity (With predator cues) | | Food intake (Without predator cues) | | Food intake (With predator cues) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wald $\chi^2_1$ | *p* | Wald $\chi^2_1$ | *p* | Wald $\chi^2_1$ | *p* | Wald $\chi^2_1$ | *p* | Wald $\chi^2_1$ | *p* |
| Pesticide (Pest) | 0.422 | 0.516 | 5.024 | **0.025** | 8.536 | **0.003** | 36.981 | **<0.001** | 27.022 | **<0.001** |
| Urbanisation (Urban) | 0.139 | 0.709 | 1.940 | 0.164 | 0.559 | 0.455 | 1.009 | 0.315 | 0.830 | 0.362 |
| Temperature (Temp) | 0.904 | 0.341 | 2.457 | 0.117 | 0.429 | 0.512 | 7.898 | **0.005** | 0.355 | 0.552 |
| Pest × Urban | 0.171 | 0.679 | 4.785 | **0.029** | 5.311 | **0.021** | 0.191 | **<0.001** | 0.672 | 0.379 |
| Pest × Temp | 3.887 | **0.049** | 1.525 | 0.217 | 1.131 | 0.288 | 22.556 | 0.070 | 0.773 | 0.412 |
| Urban × Temp | 1.020 | 0.312 | 1.227 | 0.268 | 1.845 | 0.174 | 7.756 | 0.662 | 1.945 | 0.627 |
| Pest × Urban × Temp | 0.278 | 0.598 | 0.044 | 0.834 | 1.123 | 0.289 | 3.285 | **0.005** | 0.236 | 0.163 |
| Body mass | 2.078 | 0.149 | 0.132 | 0.716 | 0.193 | 0.661 | 84.304 | **<0.001** | 22.500 | **<0.001** |

*Activity, food intake and boldness*

The effect of the pesticide on activity (both with and without predator cues) differed between urban and rural larvae (Urbanisation level × Pesticide, Table 2, Fig. 2C-D). When previously exposed to the pesticide, rural larvae tended to decrease their activity (Tukey test pooled across temperatures: without predator cues, $p$ =0.099; with predator cues, $p$ = 0.001), while urban larvae instead increased their activity (without predator cues, $p$ = 0.022; with predator cues, $p$ = 0.003). Temperature, nor its interactions had a significant effect on these behaviours.

As for activity, the effect of the pesticide on food intake in the absence of predator cues differed between urban and rural larvae (Pesticide × Urbanisation level, Table 2). Moreover, the pesticide effect was further modulated by temperature generating a Pesticide × Urbanisation level × Temperature interaction (Table 2, Fig. 2E-F). Previous pesticide exposure reduced the food intake in rural larvae at both temperatures (Tukey test: both $p < 0.001$), whereas for urban larvae this effect was only significant at 24°C ($p = 0.003$), but not at 20°C ($p = 0.909$). When tested in the presence of predator cues, food intake differed significantly only between pesticide treatments: pesticide-exposed larvae showed lower food intake rates (Table 2, Fig.2E-F). Temperature and urbanisation level had no significant effect on food intake with predator cues (Table 2, Fig. 2E-F).

Both boldness measures, i.e. the relative change in grid movements and in food intake to the presence of predator cues, did not depend on any of the treatments (grid movements: all $p > 0.08$; food intake: all $p > 0.1$).

## Discussion

While the ecologically relevant CPF concentration did not affect survival and growth rate, most of the behaviours were affected by previous pesticide exposure. Moreover, the pesticide effect on several behaviours was modulated by the urbanisation level and temperature. We here discuss these effects in more detail, thereby paying particular attention to the striking differences in the responses to the pesticide between urban and rural larvae.

The lack of effects of CPF on survival indicates that we used sublethal pesticide levels in the current study. Also the other life history trait studied, growth rate during the exposure period, was not negatively affected by the pesticide, and this in contrast to studies that documented a negative effect of exposure to similar chlorpyrifos concentrations on related coenagrionid species (e.g. Dinh Van et al. 2013, 2014a). Notably, the absence of a CPF-induced growth reduction occurred while we did observe a negative effect of the pesticide on food intake. Probably, the efficiency to convert ingested food into body mass increased in the presence of the pesticide (as observed in the study species when exposed to atrazine and endosulfan, Campero et al. 2007), and thereby kept the growth rate independent of the presence of the pesticide. On the other hand, it might be possible that feeding rate only started to be affected by the pesticide after prolonged exposure (in our case, towards the end of 15-day pesticide exposure period), so that the effect of reduced feeding rate on growth rate was not yet captured (delayed effects, Reinert et al. 2002).

While previous pesticide exposure did not affect exploration behaviour at 20°C, it was associated with an increased exploration behaviour at 24°C. The fact that CPF-exposed larvae increased exploration at the high temperature could be a reaction to the increase in the toxicity of the CPF treatment at higher temperatures (Lydy et al. 1999; Bednarska et al. 2009; for coenagrionid damselfly larvae, see Janssens and Stoks 2013b; Dinh Van et al. 2014a, 2014b). This is especially expected as 24°C seemed a suboptimal temperature for the *C. puella* larvae, indicated by the higher mortality at 24°C than at 20°C. Part of the higher toxicity of the CPF treatment at 24°C may have been caused by the higher formation of 3,5,6-trichloropyridinol (TCP), the primary metabolite of chlorpyrifos (Howard et al. 1991). TCP has been shown to be more toxic than CPF and, moreover, may interact synergistically with CPF (Cáceres et al. 2007). Therefore, at 24°C CPF-exposed larvae might have been exploring more actively in order to search for places with higher food densities (in aquatic systems food is often patchy, Chase et al. 2001) to meet the higher energy demand for detoxification and repair. We indeed have documented that at the used CPF concentration, coenagrionid damselfly larvae show an increase in the levels of the stress protein Hsp70 and in activity of the detoxification enzyme glutathione-S-transferase (Janssens and Stoks 2013a), both involved in physiological defence mechanisms.

A key finding of our study was that rural and urban larvae showed consistently different, sometimes even opposite, behavioural responses to pesticide exposure for three ecologically relevant traits: activity (both with and without predator cues) and food intake without predator cues. In agreement with previous studies, CPF had an overall negative effect on both mobility (as measured by grid movements) and food intake (Dell'Omo 2002; Dinh Van et al. 2014a, 2014b) in rural larvae. This reduction of behaviours has been explained by the CPF-induced inhibition of acetylcholinesterase which disturbs nerve transmission, and may eventually impair muscle activity (e.g. Printes and Callaghan 2004; Xuereb et al. 2009). This seems, however, less likely in the current study given no similar effect was observed for exploration behaviour. More likely, lowered levels of energy storage molecules may have prevented high activity levels (see Mikolajewski et al. 2005).

In contrast to rural larvae, when exposed to CPF urban larvae increased activity (both with and without predator cues), and kept food intake without predator cues constant (at 20°C). This seems to suggest that urban larvae were less affected by CPF, as they could avoid a reduction in food intake. As for exploration, the higher mobility may reflect more intensive searching for places with higher food density to satisfy their likely increased energy need due to increased detoxification and repair processes. Note that at 24°C, and also in the presence of predator cues, urban larvae could no longer keep food intake constant after pesticide exposure and reduced food intake; a similar pattern was also observed for rural larvae. This is consistent with the observation that CPF has been shown to be more toxic at higher temperatures, and that pesticides such as CPF may affect the way predator cues are perceived (Dinh Van et al. 2014a, 2014b). This also indicates that there was no signal of thermal adaptation counteracting the increased toxicity of CPF at higher temperature in the studied urban populations. This may not be that surprising, as also along a large-scale latitudinal gradient no such effects of thermal adaptation were detected for the vulnerability to CPF in another coenagrionid damselfly (Dinh Van et al. 2014b).

The reason for the apparent lower vulnerability to CPF in urban compared to rural larvae needs further investigation, but is in line with the expected higher contaminant levels in urban areas (Gilliom 2007). Due to more intense human activity, it is reasonable to assume that urban ponds are exposed to higher concentrations of pesticides compared to the here studied rural ponds that were all situated in nature areas and distant from urbanised land. Urban areas are distinguished by hard surfaces such as concrete and asphalt, which facilitate surface runoff that may contain contaminants (Jiang et al. 2010). In urban streams, concentrations of pesticides have indeed been shown to frequently exceed thresholds for the protection of aquatic biota (reviewed in Paul and Meyer, 2001). On the other hand, lower vulnerability to CPF in urban compared to rural larvae could be a result of cross-tolerance: studies show that increased tolerance to a certain pesticide can induce increased tolerance to other pesticides that have similar modes of action (e.g. Hua et al. 2013), and even to pesticides with different modes of action (e.g. Hua et al. 2014). Along with urban areas, agricultural landscapes are also associated with intense pesticide use. It has been shown that the pesticide tolerance levels of natural populations are correlated with the proximity of the ponds to arable land (Cothran et al. 2013) and the percentage of agricultural land coverage surrounding the pond (Coors et al. 2009). Moreover, Declerck et al. (2006) reports a similar correlation between the "quality" (i.e. clear water conditions and vegetation complexity) of the pond and the presence/absence of crop land in a 20 m radius (negative effect), and percentage of land covered by forest in a 200 m radius (positive effect). As none of our rural ponds were in the immediate vicinity of agricultural land (min. distance 110 m), and all of them were surrounded by woodland, we do not expect elevated pesticide tolerance in the studied rural ponds due to agriculture. Note that any such (unlikely) effects of agriculture in the studied rural populations would have made our finding of a lower vulnerability to CPF in urban compared to rural larvae conservative.

Whatever the underlying reason, the here observed striking differences in CPF-induced effects on behaviours between rural and urban larvae are likely general, to have a genetic base and to translate into fitness consequences. Indeed, the differences between rural and urban larvae were consistent across three urban and three rural populations reared under common-garden conditions from the egg stage. While we started with field-collected mothers, we cannot fully exclude a contribution of maternal effects. Yet, maternal effects are shown to be small in damselfly larvae (Strobbe and Stoks 2004; Shama et al. 2011). Moreover, behaviours were tested when larvae had been reared for ca. 175 days under common garden conditions, and maternal effects are known to decay with age (Lindholm et al. 2006). The fitness consequences of increased activity levels in pesticide-exposed larvae will depend on their relative benefits and costs. On the one hand, the higher mobility may lead to higher food intake by increasing encounter rates with high-quality food patches. On the other hand, the higher mobility will also increase encounter rates with predators (Werner and Anholt 1993; Brodin and Johansson 2004; for the study species: Stoks and Johansson 2000).

As shown before, the damselfly larvae reduced their activity levels under predation risk in order to avoid being detected (e.g. Brodin and Johansson 2004; Stoks et al. 2003, 2012; for the study species: Stoks and Johansson 2000; Mikolajewski et al. 2005). These behavioural responses to predator cues (i.e. boldness) were, however, not affected by previous exposure to the pesticide. In a study investigating behavioural responses to pharmaceuticals, boldness was reported to be the only behaviour not affected by the low-concentration treatment (Brodin et al. 2013), although at high concentrations exposed fish became bolder. This further illustrates that different behaviours may respond differently to pesticides, and that ecotoxicological studies focusing on a single behaviour may be misleading.

## Conclusions

Despite the rapidly increasing urbanisation levels worldwide (UN 2014), urbanisation is a largely overlooked spatial component in ecotoxicology. Our findings suggest that the ecological impact of the here used sublethal CPF exposure may critically differ between urban and rural populations, and this partly depending upon temperature, and therefore may be an important factor to consider to arrive at a spatially explicit ecological risk assessment (Van den Brink 2008; Clements et al. 2012). The level of local urbanisation may be an ignored reason why studies on the same species using different source populations may generate sometimes widely different vulnerabilities to pesticides (e.g. Naylor et al. 1990). Future studies should focus on the underlying reasons for the here documented striking urbanisation-related differences in pesticide effects on ecologically relevant traits. Another promising avenue for further research would be including other abiotic factors that may also consistently differ between urban and rural ponds (such as water turbidity), and thereby further modulate the urbanisation-specific vulnerability patterns to pesticides.

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# CHAPTER 3

# Pesticide-induced changes in personality depend on the urbanisation level

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## Abstract

Globally increasing urbanisation causes major anthropogenic changes in ecosystems, drastically altering phenotypes of organisms. Increased contamination is a well-known result of urbanisation, and its effect on behaviour has been extensively studied. Yet, animal personality, consistent behavioural variation between individuals, has rarely been investigated in the context of anthropogenic contaminants. Changes in personalities may affect the viability of populations, and even alter community dynamics. We investigated the effects of exposure to a sublethal dose of the commonly used pesticide esfenvalerate on two personality traits, activity and boldness, and compared these effects between replicated rural and urban populations using larvae of the damselfly *Coenagrion puella*. We tested for effects on behaviour at three distinct levels: the average levels of behaviours, the consistency of behaviours (repeatability), and the structure of the behavioural correlations (behavioural syndrome). We found that the pesticide treatment changed the average activity and the behavioural covariation (activity and boldness), but not the behavioural repeatability. Importantly, these pesticide-induced patterns depended strongly on urbanisation level. The average activity reduction due to pesticide exposure was only present in urban individuals. Moreover, while a behavioural correlation between activity and boldness in rural larvae appeared only after the pesticide treatment, this activity–boldness syndrome was consistently present in the urban larvae. These differential responses of urban and rural populations may be explained by the apparently more efficient coping mechanism with contaminants of urban populations, as well as the generally more stressful urban habitats. These results highlight the importance of measuring behavioural expressions at various levels when assessing contaminant effects, and not just the means. Further, we suggest that pollution may play an important role in understanding the evolution and maintenance of animal personalities in natural populations.

## Introduction

Urbanisation is a major anthropogenic process (Seto et al. 2012), causing rapid phenotypic change among a wide range of taxa (Alberti et al. 2017a). One result of urbanisation is an increase in contamination of aquatic habitats (e.g. Gilliom 2007; Hayzoun et al. 2014; Wang et al. 2016), mainly driven by the increased runoff due to the impervious surface cover typically found in urban areas (Jiang et al. 2010; Paul and Meyer 2001). Contaminants are well known to alter behaviour (Dell'Omo 2002; Tuomainen and Candolin 2011; Zala and Penn 2004). Studying behavioural responses to contaminants is important, as changes in behaviours may have crucial ecological consequences (Pyle and Ford 2017). Only recently have effects of contaminants been shown to go beyond changing mean levels of behaviours to include changing individual variation in behaviours (Montiglio and Royauté 2014).

Personality is defined as consistent behavioural variation between individuals (Réale et al. 2007), and has been documented in diverse taxa (Wolf et al. 2008), including invertebrates (Kralj-Fišer and Schuett 2014). Central to the study of personality is the concept of repeatability, the consistency of a single behaviour over time and context (Réale et al. 2007). Behavioural correlations (including those between nonpersonality traits) are referred to as behavioural syndromes (Sih et al. 2004a), and are frequently integrated into personality literature (Réale et al. 2007). Populations of the same species have been shown to differ in terms of both repeatability and behavioural syndromes (Bell 2007; Sih et al. 2004, 2012). Evidence accumulates that this differentiation is linked to environmental conditions to which the populations are exposed (e.g. predation pressure; Dingemanse et al. 2007), including anthropogenic impacts (e.g. pesticide application; Royauté et al. 2014). A change in personality structure is important for the viability of populations, as differentiation in personalities can affect biotic interactions (e.g. predator–prey interactions; Belgrad and Griffen 2016; social interactions; Santostefano et al. 2016), thereby potentially altering community dynamics (Moran et al. 2017; Start and Gilbert 2017). Studies typically investigate contaminant effects only on repeatability (e.g. Kolok et al. 1998; White and Briffa 2017) or only on behavioural syndromes (e.g. Brodin et al. 2013; Royauté et al. 2014), but rarely consider both (but see Dzieweczynski et al. 2016; Royauté et al. 2015).

Exposure to contaminants may alter behavioural repeatabilities in opposite ways, driven by two contrasting feedback loops (Figure 1 in Montiglio and Royauté 2014). On the one hand, the personality type of individuals (e.g. more active or bold personalities) may lead to an increased exposure to the contaminant, which in turn may increase the expression level of the behaviour. This positive feedback loop will cause an increase in repeatability by amplifying individual differences in behaviour. On the other hand, an increased exposure to the contaminant driven by the behavioural type may feed back negatively via the toxicity of the contaminant, decreasing the expression level of the behaviour. This would weaken individual differences in behaviour, leading to a lower repeatability. The majority of studies exploring effects of contaminants on behavioural repeatability have supported the latter prediction, i.e. a decrease in repeatability (e.g. Dzieweczynski 2011; Dzieweczynski et al. 2016; Kolok et al. 1998; Royauté et al. 2015; but see Tosetto et al. 2017; White and Briffa 2017). Behavioural syndromes may also be affected by contaminant exposure. Experimental manipulations have demonstrated that behavioural correlations may (dis)appear when animals are exposed to an environmental stressor (Barber and Dingemanse 2010; Bell and Sih 2007; Royauté et al. 2015; Snekser et al. 2009). It is suggested that certain combinations of behaviours are selected for as a result of different selection pressures (Bell 2005; Sih et al. 2004a; Sih et al. 2012). A frequent finding is the appearance of correlations of previously uncorrelated behaviours in stressful situations (e.g. predation pressure: Bell and Sih 2007; contaminant exposure: Brodin et al. 2013; low-quality habitat: Snekser et al. 2009), yet the exact causes of and mechanisms influencing behavioural syndromes still remain poorly understood.

Because of its link with anthropogenic stressors, the level of urbanisation has also been associated with differences in personality and behavioural syndromes. Anthropogenic disturbances such as increased temperature anomalies (e.g. Benz et al. 2017; Jones et al. 2008), increased pulses of pollution (e.g. Gilliom 2007; Hayzoun et al. 2014) and altered food sources (e.g. Ditchkoff et al. 2006; Murray et al. 2015) are frequently associated with urbanisation. This suggests urban habitats are more unpredictable than natural rural habitats (Parris 2016). Anthropogenic disturbances in general are expected to influence behavioural repeatabilities and syndromes (Killen et al. 2013, 2016; Sih et al. 2012). Although lower repeatabilities (Kralj-Fišer and Schneider 2012) and increased behavioural flexibility (Lowry et al. 2013) have been suggested to be beneficial for coping with the unpredictable nature of urban habitats, the only explicit study on this found no difference between repeatabilities of rural and urban populations of the European blackbird, *Turdus merula* (Miranda et al. 2013; but see preliminary results by Charmantier et al. 2017 indicating lower repeatability in urban great tits, *Parus major*).

Behavioural syndromes, on the other hand, frequently differ between rural and urban populations (Miranda et al. 2013). Most empirical evidence comes from studies with birds, and indicates weaker behavioural correlations in urban populations (Evans et al. 2010; Riyahi et al. 2017; Scales et al. 2011) due to assumed weaker selection pressures in urban habitats (e.g. abundant food resources, fewer predators; Shochat et al. 2006). Finally, there is ample evidence from studies on vertebrates for urbanisation-related differences in mean levels of personality traits. The emerging pattern points towards higher boldness in urban individuals (Lapiedra et al. 2016; Samia et al. 2015), assumed to be caused by the adaptation to human presence or relaxed predation pressure in urban populations (Miranda et al. 2013; Samia et al. 2015; Sih et al. 2011; Sol et al. 2013). Yet, it is not clear how invertebrates respond behaviourally to urbanisation.

We investigated the effects of exposure to a sublethal dose of a pesticide on personality traits and their covariation, and compared these effects between rural and urban populations using damselfly larvae as study organisms. We studied activity and boldness, two commonly used fitness-related personality traits (Réale et al. 2007; Smith and Blumstein 2008) that typically form a syndrome with more active animals being bolder (Réale et al. 2007). We tested for effects of the pesticide not only on the average levels of behaviours, but also on the consistency of the behaviours (i.e. repeatability) and the behavioural correlations (i.e. behavioural syndrome). We used replicated rural and urban populations of the damselfly *Coenagrion puella* (abundant in ponds in both rural and urban areas; Goertzen and Suhling 2013), which shows local adaptation to urban ponds in its behavioural response to pesticides (Tüzün et al. 2015). We further tested whether a pesticide-induced change in the different levels of the behaviour (i.e. average, repeatability and behavioural syndrome) predictably depends on the level of urbanisation of the populations. As aquatic insects are not expected to habituate to humans, and predation pressure does not differ between the rural and urban ponds studied in this experiment (Tüzün et al. 2017a), we did not predict any distinct differentiation in mean levels of behavioural traits between rural and urban damselfly larvae. Instead, we predicted that individuals inhabiting urban areas would show a lower behavioural consistency, i.e. lower repeatabilities, as this is expected to help populations cope with the unpredictable nature of urbanised habitats (Kralj-Fišer and Schneider 2012). As weaker selection pressures have been shown to relax behavioural correlations (e.g. Bell and Sih 2007; Scales et al. 2011), we predicted more stable correlations across contexts (i.e. from pre-exposure to postpesticide exposure periods) in population inhabiting the more stressful urban areas. As for the effects of the pesticide, we predicted a decrease in repeatability after pesticide exposure due to the suggested negative feedback between behaviour and contaminant exposure (Montiglio and Royauté 2014), stronger behavioural correlations due to the imposed stressful conditions (as in Brodin et al. 2013), and a decrease in mean levels of behaviours (Dinh Van et al. 2016).

## Material and methods

### *Study species and populations*

*Coenagrion puella* is one of the commonest damselflies in Europe (Dijkstra and Lewington 2006). Adult *C. puella* reproduce in early summer, and larvae hatch from eggs ca. 3 weeks later. Larval development takes ca. 10 months (Lowe et al. 2009). We studied three rural populations (Bierbeek, Bornem and Houwaart) and three urban populations (Leuven, Mechelen and Oudenaarde), all situated within a 45 km radius in Flanders, Belgium. Sampled ponds were shallow water bodies with abundant aquatic vegetation. We based our selection of rural and urban ponds on a two-step procedure using the geographical information system (GIS). We used % built-up area as a proxy for the urbanisation levels: we classified rural plots as <3% built-up area and urban plots as >15% built-up area. Applying these criteria, we first selected three rural plots and three urban plots of 3 x 3 km. Next, we selected a subplot of 200 x 200 m in each of the 3 x 3 km plots with the same urbanisation level to ensure that both the direct environment and the broader surroundings reflected the same urbanisation level. This sampling design has been applied in several recent studies on the effects of urbanisation on animals (Kaiser et al. 2016; Piano et al. 2017; Tüzün et al. 2015). Note that the chosen rural populations were not in direct contact with croplands; hence we did not expect intense contamination in rural ponds from agricultural activity (see Tüzün et al. 2015 for a more detailed discussion).

During June 2014, we collected mated females from each of the six populations and transferred them to the laboratory where we provided them with wet filter paper for oviposition. Hatchlings were kept in the laboratory and fed daily *Artemia* nauplii ad libitum. After 3 weeks, larvae were transferred to microcosms (10-litre plastic containers) situated in an outdoor experimental area. We used eight replicated microcosms per population (total of 48 microcosms), with 30 larvae in each microcosm (total of 1440 larvae). Larvae were provided ad libitum with *Daphnia* spp. For the current study, we tested personality traits in a subset of larvae, whereas the other larvae were part of another study focusing on life history traits. Specifically, in April 2015 we selected 60 larvae (10 per population) in their penultimate instar from the microcosms (one to two larvae per microcosm). Sampled larvae were moved to a nearby indoor facility where they were housed individually in plastic rearing containers (10.5 x 7.5 cm and 2.5 cm high) with 100 ml of dechlorinated tap water, and fed daily ad libitum with *Daphnia* spp. We checked daily for moults into the ultimate instar.

### *Experimental protocol*

Behavioural trials were conducted between 8 May and 3 June 2015 following the scheme shown in Fig. 1. Larvae were tested for their activity and boldness three times (every other day) during a 4-day ‘pre-exposure’ phase. Then on day 5, half of them were exposed to a pesticide pulse. Four days later, on day 9, we started a 4-day ‘postexposure’ phase, where larvae were again tested for their activity and boldness three times (every other day). This resulted in a total of six trials per individual (total of 360 trials) across the entire 12-day experimental period. We tested larvae in four ‘cohorts’ of 12–20 individuals of similar age. Larvae in each cohort were 6–10 days in their ultimate instar when tested for the first time

Per trial, larvae to be tested were moved to observation containers (new containers with the same dimensions and water level as the rearing containers) 24 h prior to trials. This was done both to minimize micro-environmental variation between containers (e.g. due to algal growth in the rearing containers) and to obtain high-quality video recordings to facilitate accurate behavioural scoring (see Behavioural scoring). The sides of the containers were covered with tape to prevent visual contact with conspecifics (damselfly larvae are cannibalistic; De Block and Stoks 2004). Larvae were starved 24 h prior to the behavioural assays to ensure equal hunger levels. Containers were placed in a circulating water bath, where the temperature was kept at 20 °C throughout the trials with a thermostat (Julabo F10, Julabo, Seelbach, Germany). The set-up allowed up to 12 larvae to be tested simultaneously. Trials started after an acclimation time of 20 min. All trials were performed between 1400 and 1900 hours. After acclimation, we started the activity trial where we recorded larval activity for 10 min using a video camera (Sony HDR-CX240) located above the containers. After 10 min, we started the boldness trial: we disturbed the larvae by touching their lamellae with a plastic probe until they initiated an escape response, and recorded the larvae for 15 min to measure the latency to resume moving (see Behavioural scoring). To prevent carryover effects from the boldness trial, we always conducted the activity trial first (Bell 2013). After the boldness trial, larvae were moved back to their rearing containers.

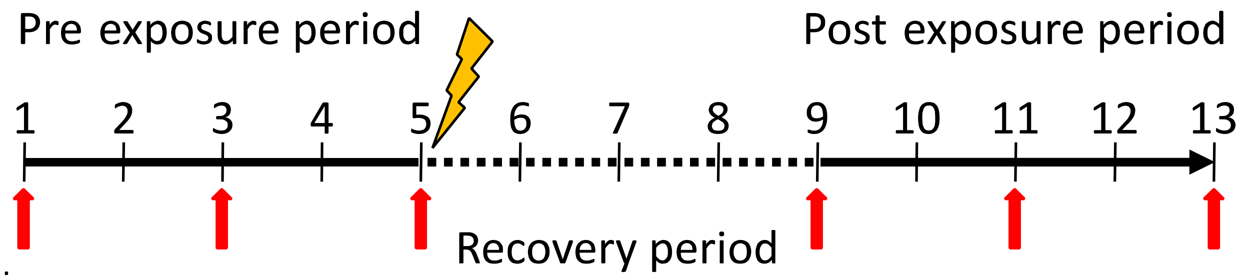

**Figure 1**. Schematic representation of the experimental design. Larvae were tested for their activity and boldness three times during a 4-day 'pre-exposure' phase. On day 5, half of them were exposed to a pesticide pulse (indicated with a bolt). After a 4-day recovery period, we started a 4-day 'postexposure' phase, where larvae were again tested for their activity and boldness three times. Days on which behavioural trials were conducted are indicated with arrows.

On day 5, after completion of the pre-exposure trials, we exposed half of the larvae in their rearing container to the pesticide esfenvalerate. This widely used pesticide is a synthetic pyrethroid that affects the nervous system by interacting with sodium channels (Narahashi 2000). We used a concentration of 0.2 µg/litre, which has been reported as sublethal for another *Coenagrion* damselfly (Dinh Van et al. 2016). This concentration is ecologically relevant as it falls within the range of concentrations detected in water bodies (Stampfli et al. 2013). We prepared a 0.1 mg/ml stock solution by dissolving esfenvalerate powder (purity > 99%, Sigma-Aldrich) in absolute ethanol. This stock solution was further diluted with dechlorinated tap water to obtain a solution with the concentration of 4 µg/litre esfenvalerate, from which 5 ml was gently poured over the surface of each container to obtain the nominal esfenvalerate concentrations of 0.2

µg/litre. The control group was treated with the same ethanol concentration as used in the pesticide treatment to dissolve the pesticide. Esfenvalerate concentrations were measured on pooled samples from 10 containers using gas chromatography equipped with an electron capture detector at the Laboratory of Crop Protection Chemistry, Ghent University (Ghent, Belgium). The initial concentration (20 min after application) was 0.054 µg/litre, and the final concentration after 14 h was 0.023 µg/litre. We restricted the pesticide exposure period to 14 h by moving larvae into new rearing containers with water. We started with the postexposure behavioural trials 4 days after the pesticide treatment, as larvae were still experiencing muscle contractions 3 days after the exposure (see Results).

We quantified the growth rate of larvae across the entire experimental period based on the increase in wet mass. After gently blotting larvae dry with tissue paper to ensure that no water remained on them, we weighed larvae to the nearest 0.01 mg using an electronic balance (AB135-S, Mettler Toledo). We did so on the first and last day of the 12-day experimental period. We calculated growth rate as [ln(body mass at day 1) – ln(body mass at day 13)]/12. We also determined the sex of each larva. Owing to a mistake, we lost the data for growth rate and sex of 10 and 8 individuals, respectively.

### *Behavioural scoring*

We measured activity levels of each larva as the distance travelled in 10 min. We did this by calculating the position changes of each larva in consecutive frames derived from the video recordings. In short, we first converted the video files (the first 10 min, which was recorded for activity scoring) into a stack of images, i.e. frames. As the videos were originally recorded at 25 frames/s (fps), which would result in an image stack too large to work with, we chose to select one frame per 2 s (corresponding to 0.5 fps). Next, using the image stack now consisting of a total of 300 frames, we created a single ‘background image’ representing nonmobile elements (e.g. the trial containers), which we subtracted from each of the 300 frames. The output of this process was an image sequence where only mobile larvae were visible. Using the Mtrack2 plug-in in the software ImageJ (Schneider et al. 2012), we quantified the distance moved (in mm) of each larva during the 10 min trial.

We quantified boldness as latency to resume moving (frequently used in behavioural studies with damselfly larvae; e.g. Brodin 2009; Brodin et al. 2014; Debecker et al. 2016; Jonsson et al. 2014). Damselfly larvae typically react to a disturbance by performing a swimming burst to escape the potential predator (Gyssels and Stoks 2005), followed by an inactive period. We scored latency to move as the time (s) between the end of the swimming burst and the moment the larva became active again. Bold individuals are expected to become active sooner and hence to show a shorter latency. Trials in which larvae did not become active within 15 min (total of 56 trials, 15.5%) were given the maximum score of 900 s.

### *Statistical analyses*

All analyses were conducted with R version 3.2.3 for Windows (R Development Core Team 2015). We used the packages lme4 (Bates et al. 2015) for mixed-effects models, nlme (Pinheiro et al. 2016) for repeatability models with constrained variances, and rptR (Nakagawa and Schielzeth 2010) to calculate repeatabilities. We used the restricted maximum likelihood estimation method for all analyses. To meet the assumptions of normality, activity level was Box–Cox transformed, whereas latency was square-root transformed.

#### Analysis of average levels of behaviour

To test for an effect of the pesticide treatment and urbanisation level on the average expression levels of behaviours, we constructed separate linear mixed-effect models per behaviour. These models included pesticide treatment (pesticide versus control), test phase (pre-exposure versus postexposure) and urbanisation level (rural versus urban), as well as their interactions as fixed effects. For example, a significant test phase*pesticide treatment*urbanisation level interaction would indicate that the effect of the pesticide treatment on behaviours was present in the postexposure phase, and this effect differed between rural and urban individuals. Sex (categorical, two levels), time of trial (continuous, range 14–19 h) and days after moult into the ultimate instar when larvae were tested first (continuous, range 6–10 days) were included as additional fixed effects to all models. Nonsignificant covariates were removed to avoid overparameterization. All models included the following random effects: larval identity nested within population (because repeated measures per individual are not independent replicates), population nested within urbanisation level, and cohort (set of larvae tested together). To further interpret significant interactions, we performed Tukey post hoc tests.

We analysed treatment effects on growth rate with a linear mixed-effect model, where we included pesticide treatment and urbanisation level and their interaction as fixed effects, and population (nested within urbanisation level) and cohort as random effects. As male and females may differ in their growth rate when exposed to pesticides (for the study species: Campero et al. 2008), we also included sex and its interaction with pesticide treatment in the models.

### Analysis of repeatability

We measured behavioural repeatabilities separately for each treatment combination as the ratio of between-individual variance to the total phenotypic variance (Nakagawa and Schielzeth 2010). To assess whether the treatments affected repeatabilities, we compared models where between- and within-individual variance (i.e. the two components used to calculate repeatability) were constrained to be equal among treatments (pesticide and control group) and urbanisation levels (rural and urban) or for each level of the combination of the two categories (e.g. urban individuals from the control group), with models where these variance components were estimated independently for each group (Dingemanse and Dochtermann 2013; see Appendix Table A1 for the list of competing models). As repeatabilities may differ between the sexes (Bell et al. 2009), and the pesticide effect on repeatability can be sex dependent (Royauté et al. 2015), we also compared models with the same structure as described above, where sex or the combination of the sex and pesticide treatment was the grouping factor. We compared these models based on their Akaike information criterion (AIC) values, with $\Delta AIC > 2$ indicating a significant difference between models (Burnham and Anderson 2002). These models included urbanisation level, pesticide treatment and sex as fixed effects to calculate an unbiased estimate of repeatability (‘adjusted repeatability’; Nakagawa and Schielzeth 2010).

### Analysis of covariation

To assess whether covariation patterns (hence the syndrome) between activity and boldness differed across treatments, we constructed a linear mixed-effect model with activity level as the response variable, and the interactive term of latency with the treatment variables as fixed effects (see Debecker et al. 2016 for a similar approach). A significant pesticide treatment*latency interaction would indicate that the strength of the covariation between activity and boldness depends on the pesticide treatment. Note that a negative covariation of activity levels and latency should be interpreted as a positive covariation of activity and boldness (lower latency indicates bolder individuals).

## Results

### *Average levels of behaviours and growth rate*

Directly after the pesticide application, all larvae exposed to esfenvalerate became fully paralysed (lying motionless on their sides) for at least several hours. All larvae recovered, yet still showed spasms up to 3 days after the pesticide application.

After the recovery period, the pesticide differentially affected the activity of rural and urban larvae (pesticide treatment*test phase*urbanisation level: $F_{1,289.7} = 7.162$, $P = 0.008$; Fig. 2a, b). Post hoc tests showed that urban larvae from the control group significantly increased their activity levels during the postexposure phase ($P = 0.001$), whereas rural larvae had similar activity levels across the pre-exposure and postexposure phases ($P = 0.767$; Fig 2a). In contrast, in the pesticide treatment group, neither urban ($P = 0.778$) nor rural larvae ($P = 0.370$) changed their activity levels (Fig. 2b). In other words, the ontogenetic increase in activity levels of urban damselfly larvae over time (from pre-exposure days 1–5 to postexposure days 9–13) was disrupted by the pesticide exposure. The same interaction also revealed that for the urban individuals, the pesticide treatment reduced activity levels when compared to the control group ($P = 0.040$), whereas in rural individuals, activity levels were not affected by the pesticide treatment ($P = 1.00$). Sex and its interactions had no effect on activity (all $P > 0.3$).

Latency (the boldness measure) did not differ between any of the treatment groups ($P > 0.20$ for main effects and all interactions; Fig. 2c, d). For growth rate, we found a significant pesticide treatment *urbanisation level interaction ($F_{1,29.2} = 5.826$, $P = 0.022$): while the control and pesticide treatment groups of rural larvae did not differ in growth rate ($P = 0.973$), urban larvae showed a decrease in growth rate when exposed to the pesticide ($P = 0.028$; Fig. 3). Male and female larvae did not differ in growth rate ($P > 0.10$ for main effect and all interactions).

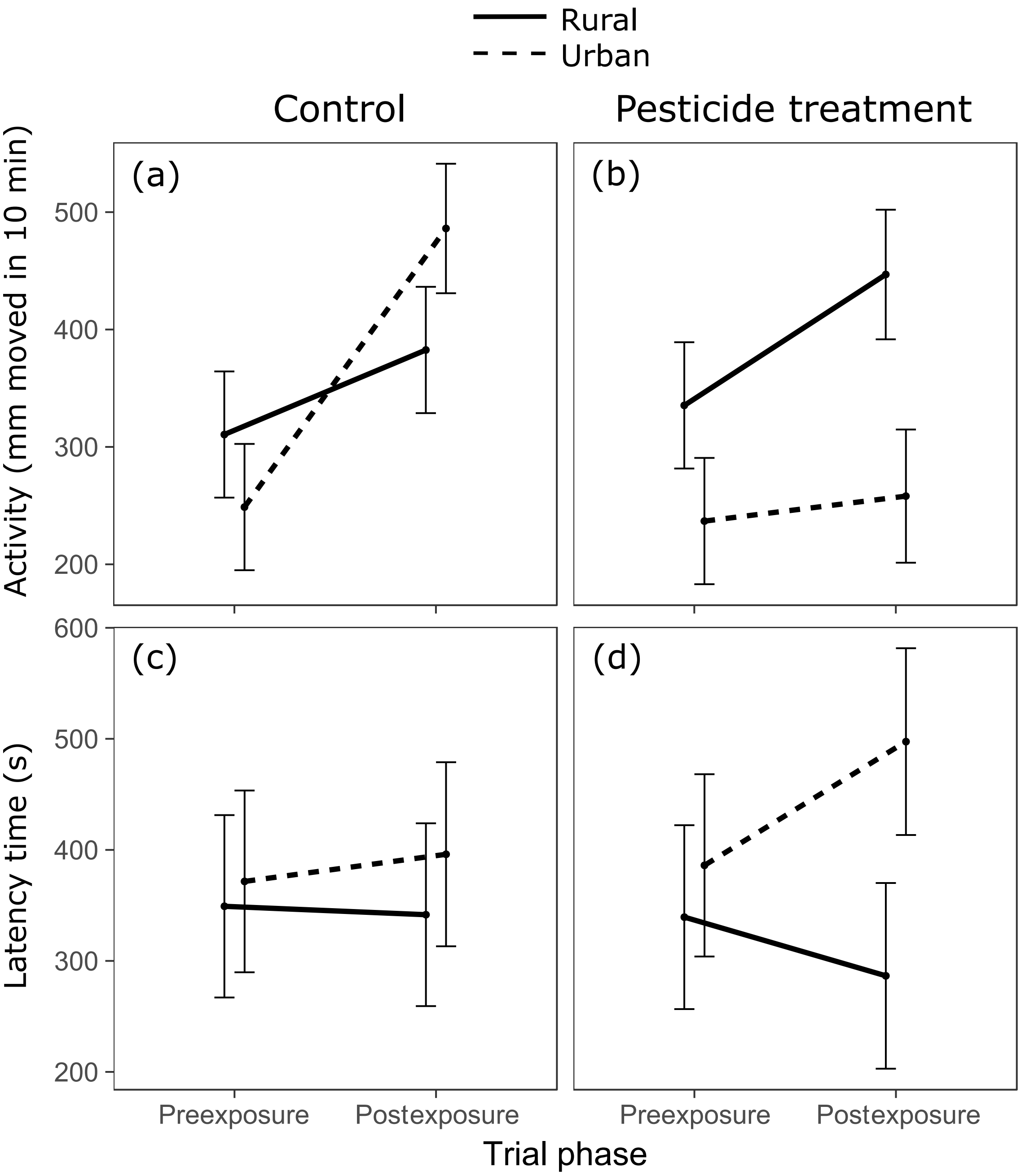

**Figure 2**. (a, b) Activity levels and (c, d) boldness of *Coenagrion puella* as a function of pesticide exposure and urbanisation level. Activity was measured as distance (mm) moved in 10 min. Boldness was quantified as latency to move (s) after a simulated predator attack. Note that higher values indicate shyer behaviour. The change in behaviour from pre-exposure to postexposure is shown separately for the (a, c) control and (b, d) pesticide treatment group. Solid and dashed lines represent rural and urban populations, respectively. Means are shown ±95% confidence intervals.

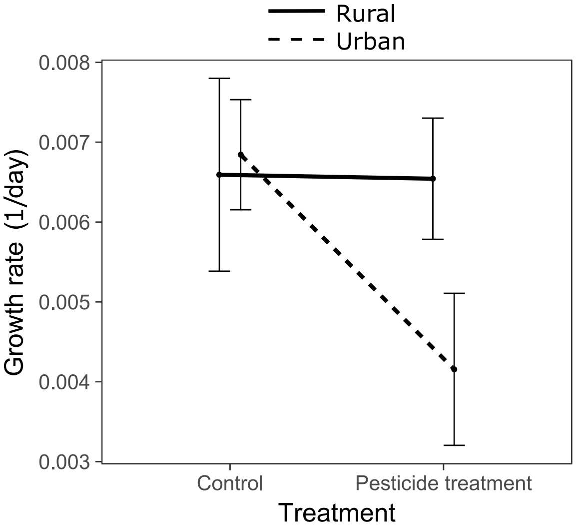

**Figure 3**. Growth rate of *Coenagrion puella* as a function of pesticide exposure and urbanisation level. Growth rate was measured for the entire 12-day experimental period. Solid and dashed lines represent rural and urban populations, respectively. Means are shown ±95% confidence intervals.

*Repeatability of behaviours*

Both activity (range 0.277–0.542) and latency (range 0.252–0.447) showed significant repeatabilities (Table 1). The model comparisons revealed that behavioural repeatabilities did not depend on urbanisation level or pesticide treatment (Appendix Table A1). Similarly, repeatabilities were consistent across sexes.

*Behavioural covariations*

The strength of the covariation between activity and latency differed strongly between the control and pesticide treatment group (pesticide treatment*latency: $F_{1,333.9} = 13.361$, $P < 0.001$), and this further depended on the urbanisation level (pesticide treatment*urbanisation level*latency: $F_{1,336.4} = 5.161$, $P = 0.024$; Fig. 4). While the nonsignificant covariation pattern between activity and latency in rural individuals of the control group (slope ± SE = -0.028 ± 0.089; $P = 0.747$) drastically turned negative with the pesticide treatment (-0.545 ± 0.102; $P < 0.001$), this change was less strong in urban individuals where there was already a significant negative covariation pattern in the control group (control group: -0.318 ± 0.116; $P = 0.008$; pesticide treatment: -0.409 ± 0.111; $P < 0.001$). Separate analyses per urbanisation level

**Table 1.** Repeatabilities (*R*) of activity and latency across treatment groups

| Behaviour | Treatment | Urbanisation level | *R* | SE | 95% CI | CV |
|---|---|---|---|---|---|---|
| Activity | Control | Urban | 0.277 | 0.118 | 0.021; 0.498 | 68.8 |
| Activity | Control | Rural | 0.542 | 0.118 | 0.277; 0.707 | 52.8 |
| Activity | Pesticide | Urban | 0.470 | 0.125 | 0.171; 0.673 | 74.0 |
| Activity | Pesticide | Rural | 0.356 | 0.123 | 0.123; 0.570 | 64.7 |
| Latency | Control | Urban | 0.357 | 0.119 | 0.112; 0.568 | 47.7 |
| Latency | Control | Rural | 0.252 | 0.114 | 0.036; 0.476 | 48.7 |
| Latency | Pesticide | Urban | 0.447 | 0.120 | 0.179; 0.639 | 41.7 |
| Latency | Pesticide | Rural | 0.420 | 0.122 | 0.159; 0.620 | 49.2 |

CI: 95% confidence intervals; CV: coefficient of variation. Estimates are derived from models with 1000 bootstraps. All repeatabilities were significant ($P < 0.001$; tested by likelihood ratio tests)

showed a significant pesticide treatment*latency interaction for rural larvae ($F_{1,168.8} = 15.223$, $P < 0.001$), but not for urban larvae ($F_{1,159.8} = 0.042$, $P = 0.838$), confirming that the change in the behavioural covariation due to the pesticide treatment was significant only in rural larvae. In other words, while activity and boldness were positively correlated in urban individuals independent of the pesticide treatment, this behavioural covariation appeared in rural individuals only after the pesticide exposure.

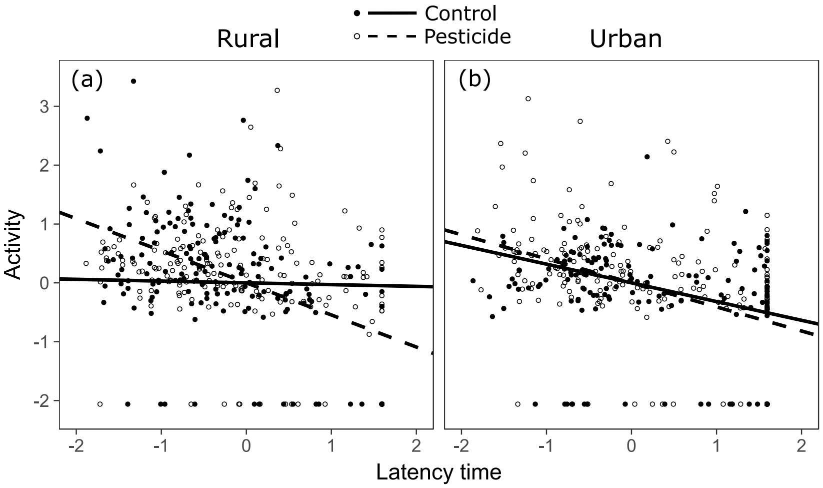

**Figure 4**. Covariation patterns between activity and boldness in response to pesticide exposure in (a) rural and (b) urban populations of *Coenagrion puella*. Regression lines are derived from mixed-effect models. Note that a negative covariation of activity and latency should be interpreted as a positive covariation of activity and boldness. Both trait values are scaled.

## Discussion

The concentration of esfenvalerate used had widespread sublethal effects by changing the mean levels of behaviour (activity), growth rate and the behavioural covariation (activity and boldness) of damselfly larvae, but did not affect the repeatability of the behaviours. Importantly, these pesticide-induced patterns strongly depended on the urbanisation level of origin. As the differentiation in personalities documented here resulted from a common garden rearing experiment, we suggest that our results reflect genetic-based (or maternal) patterns, rather than environmental differences. Below we discuss the implications of these findings, particularly focusing on possible adaptations to urbanisation.

### *Urban-rural differentiation in personality traits*

Unlike for taxa such as birds, mammals and lizards, behavioural studies comparing urban and rural populations of invertebrates are rare (Miranda et al. 2013; Sol et al. 2013), and are almost nonexistent for insects (but see Tüzün et al. 2015). A frequent finding in vertebrates is that urban individuals are bolder than their rural counterparts, mainly expressed as an increased tolerance to human presence (Miranda et al. 2013; Samia et al. 2015; Sol et al. 2013). Further, lower predation pressures in urban populations may also shape personality traits (Shochat et al. 2006), particularly driving a higher boldness (e.g. Lapiedra et al. 2016; but see e.g. Brown et al. 2005 for higher boldness in sites with high predation). Of the two noninsect arthropod studies comparing behaviours between urban and rural populations, boldness levels of the western black widow spider, *Latrodectus hesperus*, did not differ between urban and desert populations, although the authors initially predicted urban spiders to be bolder due to a lack of predators in urban habitats (Halpin and Johnson 2014). The only other study showed a weaker predator escape response in an urban population of the woodlouse *Porcellio scaber*, which was explained by the more frequent vibrations from construction and automobile traffic in urban areas (Houghtaling and Kight 2006). The latter study, however, lacked replicated populations per urbanisation level, and the experimental design of both studies did not allow the authors to determine whether this was a plastic or evolved response as they scored the behaviours directly on field-collected animals. In the present study, neither activity nor boldness differed between urban and rural populations in the absence of the pesticide, a result in agreement with previous work with the same study species (Tüzün et al. 2015). Both major drivers suggested for the above-mentioned behavioural differentiations between urban and rural populations are unlikely to hold for aquatic damselfly larvae: under water they do not habituate to human presence, and do not experience a reduced predation pressure (Tüzün et al. 2017a).

Urban and rural larvae differed in their response to the pesticide treatment, with the pesticide-induced activity and growth reduction only present in urban individuals. Exposure to esfenvalerate has previously been shown to alter behaviours in various taxa (aphids: Hurej and Dutcher 1994; honeybees: Ingram et al. 2015; fish: Little et al. 1993). The behavioural change documented here in urban larvae may be adaptive, as a lower activity (hence possibly lower metabolic rate; Biro and Stamps 2008) in contaminated waters might reduce the uptake even when the external concentrations to which larvae are exposed do not change (Montiglio and Royauté 2014). Moreover, as activity and food intake are frequently shown to correlate positively (Sih et al. 2004; in damselfly larvae: Johansson et al. 2001), reduced intake of contaminated prey may be yet another mechanism to cope with contamination in urban populations. In the present study, we excluded pesticide uptake through a dietary route by not providing damselfly larvae with food during the 14 h pesticide exposure, yet reducing food intake during contaminant exposure (independent of whether the food is contaminated or not) might be an adaptive strategy adopted by urban individuals. The reduction in growth rates with pesticide exposure observed only in urban larvae, possibly due to reduced feeding rates, supports this notion. Furthermore, urban larvae may have shifted their resource allocation when exposed to the pesticide by investing more in body maintenance (i.e. detoxification and repair) instead of growth (Campero et al. 2007). Nevertheless, we cannot fully exclude the contrasting scenario where lower larval activity may be due to a higher sensitivity to the pesticide in urban larvae. Contrary to the current finding, we have previously shown that urban individuals of *C. puella* increased their activity levels when exposed to the pesticide chlorpyrifos (Tüzün et al. 2015). The differences between the two studies may be because esfenvalerate, targeting sodium channels in the nervous system, has a different mode of action from chlorpyrifos, an acetylcholinesterase inhibitor. Furthermore, the current study adopted a more realistic shorter exposure duration (14 h versus 15 days).

### *Repeatability of personality traits*

Activity and boldness were repeatable, with the repeatability values close to the average behavioural repeatability of 0.37 obtained in a meta-analysis (Bell et al. 2009). Behavioural repeatabilities may strongly depend on the environmental conditions (e.g. temperature, pollutants, predator regime), many of which covary with anthropogenic disturbance (Killen et al. 2016, 2013), as is the case for urbanisation (Parris 2016). It has been argued that, considering the relatively more unstable urban environment (increased water pollution; Gilliom 2007; e.g. greater daily temperature fluctuations; Kaiser et al. 2016), individuals inhabiting urban areas may benefit from high within-individual variation in their behaviour (i.e. lower repeatabilities) to be able to cope with the unpredictable nature of urbanised habitats (Kralj-Fišer and Schneider 2012). Yet, we could not detect a difference in repeatability between urban and rural larvae. The only study explicitly comparing behavioural repeatabilities across urbanisation levels also showed no differentiation in the European blackbird (Miranda et al. 2013).

Repeatabilities of the personality traits of the damselfly larvae also did not change with pesticide exposure. Four of the six studies exploring effects of contaminants on behavioural repeatability (Dzieweczynski 2011; Dzieweczynski et al. 2016; Kolok et al. 1998; Royauté et al. 2015) found the predicted decrease under the negative feedback loop between behaviour and contaminant exposure (in this case presence or absence of pesticide; Montiglio and Royauté 2014; White and Briffa 2017). Under this negative feedback loop, more active individuals are expected to be more exposed to the pesticide because of their assumed higher metabolic rate (Biro and Stamps 2008). This in turn would result in a stronger decrease in activity of the more active individuals due to the negative effect of the pesticide. The end result would be an erosion of behavioural variation among individuals, and eventually translate into a lower repeatability (lower panel in Figure 1 in Montiglio and Royauté 2014). Yet, a pesticide-induced decrease in repeatability is not general, and two recent studies (Tosetto et al. 2017; White and Briffa 2017) also did not detect an effect of a pollutant on behavioural repeatability.

*Urban-rural differentiation in covariation patterns between personality traits*

As expected, we found evidence for a behavioural syndrome between activity and boldness (in damselflies: e.g. Brodin 2009; Debecker et al. 2016), yet this depended on both the urbanisation level and pesticide exposure. Indeed, evidence for more active rural larvae being bolder appeared only after the pesticide treatment, whereas this activity–boldness syndrome was consistently present in the urban larvae.

The presence of the syndrome in urban but not rural larvae in the control treatment matches the idea that syndromes are more likely to occur under stressful conditions. Both comparative and experimental studies suggest that low-quality habitats, hence with stronger selection pressures, select for tighter behavioural correlations (discussed below), especially so when behavioural consistency is the most beneficial response to a rapidly changing environment (McElreath and Strimling 2006). It is important to acknowledge that the urban-specific selection pressures experienced by organisms will strongly depend on the ecology of the species (Shochat et al. 2006). Considering the typical characteristics of urban ponds, for example elevated temperatures (aquatic ectotherms are particularly sensitive; e.g. Verberk et al. 2016) and contamination (damselfly larvae cannot avoid exposure in their aquatic habitat; Stoks et al. 2015), we argue that damselfly larvae inhabiting urban ponds are under stronger selection pressures compared to rural populations. In line with this, the frequent finding of an absence of behavioural correlations in urban populations of birds, in contrast to rural populations (Evans et al. 2010; Riyahi et al. 2017; Scales et al. 2011), is in fact in accordance with our results, as urban areas have been suggested to provide high-quality habitats for birds (e.g. abundant food, fewer predators), therefore imposing a relatively relaxed selection pressure compared to rural habitats. Similarly, Adriaenssens and Johnsson (2013) documented the emergence of a behavioural syndrome in wild brown trout, *Salmo trutta*, following a major survival bottleneck, and Snekser et al. (2009) reported that behavioural correlations in the damselfish *Stegastes leucostictus* only appeared in low-quality habitats (but see e.g. Lichtenstein et al. 2016 for no effect of food restriction on behavioural correlations). Furthermore, behavioural syndromes in threespined sticklebacks, *Gasterosteus aculeatus*, only developed after experimentally imposing predation pressure (driven by plasticity and selective predation; Bell and Sih 2007), or only existed in populations inhabiting ponds with predators (Bell, 2005; Dingemanse et al. 2007; but see Herczeg et al. 2009).

Exposure to contaminants should similarly create a stressful state, hence may also explain why in the rural populations the syndrome emerged after damselfly larvae had been exposed to esfenvalerate. In line with this, dilute concentrations of the pharmaceutical oxazepam led to the emergence of a previously absent behavioural correlation in the European perch, *Perca fluviatilis* (Brodin et al. 2013; but see Dzieweczynski et al. 2016 for an opposite pattern in Siamese fighting fish, *Betta splendens*, exposed to the pharmaceutical fluoxetine). In the only arthropod study, acute pesticide exposure resulted in the collapse of previously present behavioural correlations in the jumping spider *Eris militaris* (Royauté et al. 2015). The authors suggested this pattern was caused by the pesticide either altering physiological mechanisms controlling behavioural traits or changing energy budgets via reduced immune function. The pesticide exposure did not affect growth rates in rural populations used in our study; hence we suggest that a shift in energy allocation was not the underlying reason for the emergence of behavioural correlations documented here. In addition, assuming the decreased pesticide sensitivity in urban populations is linked to local adaptation (Tüzün et al. 2015), we predicted the behavioural correlations in urban individuals to be more consistent across the pesticide treatment. Unfortunately, the study by Royauté et al. (2015) only used populations with no history of insecticide exposure; hence we do not know whether spider populations (potentially) adapted to pesticide exposure are more stable in their behavioural correlations across contexts (i.e. pesticide absent versus present). Nevertheless, compared to rural populations, we found evidence of a more stable correlation of activity and boldness across the pesticide treatment in urban populations. On the other hand, our finding of a behavioural correlation appearing only after exposure to a stressor in rural populations, together with studies finding similar results (e.g. Brodin et al. 2013; Snekser et al. 2009), indicates that behavioural syndromes are in fact more flexible than previously speculated (Conrad et al. 2011).

## Conclusions

Urbanisation causes drastic anthropogenic changes in ecosystems worldwide (Seto et al. 2012), and one important aspect of urbanisation is the higher contamination levels in urban water bodies (e.g. Gilliom 2007; Hayzoun et al. 2014; Wang et al. 2016). Our results on the differential effects of pesticides in urban and rural populations have two important implications for ecological risk assessment (ERA) of pesticides. First, the response in mean levels of activity and growth rate to pesticides differed between urban and rural populations, supporting the need to include spatial variation into ERA (Clements et al. 2012; Van Den Brink 2008). Given that ecotoxicological tests using field animals typically sample rural populations, this may bias the ERA. Second, while ecotoxicological studies provide extensive information on effects of pollutants on average behavioural responses (Dell'Omo 2002), we have an extremely limited understanding of how behavioural (co)variation patterns are influenced (Montiglio and Royauté 2014). Repeatability and behavioural syndromes have only very recently begun to receive attention in the context of anthropogenic contaminants (Brodin et al. 2013; Dzieweczynski et al. 2016; Montiglio and Royauté 2014; Royauté et al. 2015; White and Briffa 2017). We demonstrated here that in rural populations, exposure to esfenvalerate generated a syndrome between activity and boldness. This is important for ERA, as it demonstrates both an example of intraspecific differentiation in vulnerability to pesticides and the importance of accounting for suites of behavioural traits, instead of focusing on a single behaviour. Personalities can shape biotic interactions such as predator–prey interactions (e.g. Belgrad and Griffen 2016; Pruitt et al. 2012) and social interactions (e.g. Briffa et al. 2015; Santostefano et al. 2016), and can even alter community dynamics (Modlmeier et al. 2015; Moran et al. 2017; Start and Gilbert 2017). Hence, we suggest that effects on personality structure to be an unexplored pathway to investigating how anthropogenic contaminants may affect ecosystems.

There is ongoing debate about what maintains variation in personality within and across populations (e.g. McElreath et al. 2007; Sih et al. 2015; Wolf et al. 2008). One important mechanism is the feedback loop between the state and the behaviour of animals (Montiglio and Royauté 2014; Sih et al. 2015). Given the widespread and steadily increasing pollution of our planet (Bernhardt et al. 2017), and that pesticide exposure can be seen as a state variable that individuals experience (Montiglio and Royauté 2014), pollution may play an important role in our understanding of the evolution and maintenance of animal personalities observed in natural populations (Montiglio and Royauté 2014; Sih et al. 2015).

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## Appendix

**Table A1.** Model comparisons for testing differences in repeatability between groups

| Model | Individual variance components differ between groups | Activity | | Latency | |
|---|---|---|---|---|---|
| | | AIC | ΔAIC | AIC | ΔAIC |
| 1 | - | 2065.544 | 0 | 2067.035 | 0 |
| 2 | Pesticide treatment (Control/Pesticide) | 2071.286 | 5.742 | 2071.373 | 4.338 |
| 3 | Urbanisation level (Urban/Rural) | 2071.132 | 5.588 | 2072.759 | 5.724 |
| 4 | Sex (Male/Female) | 2070.474 | 4.93 | 2070.161 | 3.126 |
| 5 | Pesticide treatment*Urbanisation level | 2080.223 | 14.679 | 2088.794 | 21.759 |
| 6 | Pesticide treatment*Trial phase | 2078.367 | 12.823 | 2081.668 | 14.633 |
| 7 | Pesticide treatment*Sex | 2074.425 | 8.881 | 2085.183 | 18.148 |
| 8 | Trial phase*Sex | 2079.709 | 14.165 | 2081.827 | 14.792 |

Competing models differ in the way they estimate between- and within-individual variance. For example, model 2 allows the individual variance components to differ between the control and the pesticide treatment group. Akaike information criterion (AIC) scores and ΔAIC (difference between a given model's AIC and the lowest AIC) are given separately for models with activity or latency as response variable. Note that model 1 (where individual variance components do not differ between groups) is the most parsimonious models for both activity and latency. All mixed-effect models include pesticide treatment, urbanisation level and sex as fixed effects, and individual identity as random effect.

## CHAPTER 4

# Carry-over effects across metamorphosis of a pesticide on female lifetime fitness strongly depend on egg hatching phenology: a longitudinal study under seminatural conditions

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Slightly modified version

## Abstract

Current ecological risk assessment of pesticides fails to protect aquatic biodiversity. For the first time, we tested two potential reasons for this failure with regard to carry-over effects across metamorphosis: their dependence on hatching period, and the lack of studies quantifying adult fitness under seminatural conditions. Using the damselfly *Coenagrion puella* sampled from six populations, we designed an outdoor longitudinal one-year study starting from the egg stage. We exposed the aquatic larvae to the pesticide esfenvalerate (0.11 µg/L) during the initial microcosm part. Next, we monitored the lifetime fitness of the terrestrial adults in an insectary. Exposure to the pesticide negatively impacted not only larval traits, but also drastically reduced lifetime mating success of adult females. The impact of this post-metamorphic effect of the pesticide on the population level was three times more important than the effects in the larval stage. Importantly, this carry-over effect was only present in females that hatched early in the season, and was not mediated by metamorphic traits (age and mass at emergence). We provide proof-of-principle under seminatural conditions for two potential pitfalls that need to be considered when improving risk assessment: carry-over effects on adult fitness can (i) be much more important than effects during the larval stage and may not be captured by metamorphic traits, and (ii) be strongly modulated by egg hatching dates.

## Introduction

Improving ecological risk assessment of pesticides is a major challenge as it currently fails to protect aquatic biodiversity (Beketov et al. 2013; Stehle and Schulz 2015). One possible reason for this failure is that pollutant effects may persist after metamorphosis in the many animals with a complex life cycle (Debecker et al. 2017; Wesner et al. 2014). The fate of a pollutant-exposed population heavily depends on lifetime fitness values of its individuals, and cannot be accurately judged by pre-adult traits (Forbes and Calow 2002). Studies of pollutant effects across metamorphosis, however, rarely explicitly quantified adult fitness components (but see Debecker et al. 2017). Instead, they focused on metamorphic traits such as age and mass at metamorphosis which do not accurately predict the effects of larval stressors on adult fitness (De Block and Stoks 2005; Earl and Whiteman 2015; Van Allen et al. 2010).

Another major factor limiting risk assessment is that studies on carry-over effects of pesticides are mostly limited to one population sampled at one moment in time. As a result, the widespread temporal variation in egg hatching dates within populations (De Block and Stoks 2005; Orizaola et al. 2013) is neglected in ecotoxicology. The time constraints imposed on late-hatching larvae can, however, strongly modify carry-over effects of ecological stressors (De Block and Stoks 2005; Orizaola et al. 2013; Orizaola et al. 2016; Stoks et al. 2006). This is usually assumed to be a result of reallocation of resources due to the accelerated growth and development imposed by time constraints.

To advance our insights in pesticide-induced carry-over effects, we carried out a longitudinal one-year study starting from the egg stage where we exposed the aquatic larvae of a damselfly to a pesticide pulse and studied not only effects during the larval stage and at metamorphosis, but also explicitly quantified fitness after metamorphosis in the terrestrial adult stage. To specifically assess the importance of within-season temporal variation modulating carry-over effects, we exposed in each of the six sampled populations two cohorts that differed ca. 40 days in their natural hatching period. Given that effects of pesticides are ideally measured under conditions as natural as possible (Boone et al. 2014), and to obtain realistic estimates of fitness (cfr. Tigreros 2013), we integrated an outdoor microcosm part for the larval stage with a large outdoor insectary part for the adult stage. As pesticide, we chose the pyrethroid insecticide esfenvalerate, one of the most widely applied pyrethroids (Beketov and Liess 2005; Spurlock and Lee 2008) that can cause carry-over effects in damselflies (Dinh Van et al. 2016; Janssens et al. 2017). We applied three pulses (with three-day intervals) of esfenvalerate with a concentration of 0.11 µg/L. As study species we chose the damselfly *Coenagrion puella* which is common in Europe (Dijkstra and Lewington 2006), and known to react to time constraints by accelerating growth and development rates (Mikolajewski et al. 2015).

## Materials and methods

### Study species and populations

To capture regional variation in life history, we sampled damselflies from six populations, situated in both urban and rural settings in Flanders, Belgium. We collected eggs from each of the six populations in each of two periods, 13 – 22 June ("early period"), and 23 – 31 July 2014 ("late period"), matching the peak and the end of the flight season in Flanders, respectively. This resulted in two cohorts differing ca. 40 days in egg hatching time. Given the average lifespan of adult *C. puella* is ca. 20 days (Banks and Thompson 1985; see also Results), the two collected cohorts derived from parents that do not overlap in flight period. We collected eggs from 5-10 mated females from each pond in each period. Eggs from the early and late periods hatched during 4 – 12 July and 16 – 19 August 2014, respectively. To increase survival, we reared the larvae in laboratory conditions for the first three weeks. Larvae were kept per female together in plastic containers filled with 1.5 L of dechlorinated tap water in a temperature-controlled room at 20 °C with a photoperiod of 14:10 h light:dark. Larvae were fed *Artemia* nauplii *ad libitum* five days a week.

Our initial aim was to also test whether the urbanisation level of the damselflies (urban vs rural) influenced the carry-over effects. Yet, as neither the main effect of urbanisation, nor its interaction with hatching period or pesticide exposure had any influence on the tested traits in females (see Appendix S1), we pooled individuals of the two habitat type (urban and rural), and do not report or discuss the urbanisation aspect of the study in this article.

### General experimental procedure

Following the three weeks period in the laboratory, the longitudinal one-year study consisted of two integrated outdoor parts. In the first, outdoor microcosm part, we set up a full factorial experiment consisting of 2 hatching periods (early and late) × 2 pesticide treatments (control and 0.2 µg/L esfenvalerate). This part ran from summer 2014 until spring 2015 (with a short indoor part to avoid winter freezing; see below). The pesticide application was carried out in May 2015, which is within the main application period of agricultural pesticides in the study region (Van Drooge et al. 2001). During this period all animals were still in the larval stage. As in nature, all larvae were simultaneously exposed to the pesticide irrespective of their egg hatching date the previous summer. In the second, outdoor insectary part, adults that emerged from the microcosms were placed in a single outdoor insectary where we monitored adult life-history traits and lifetime mating success. This part ran from late spring to summer 2015.

### Outdoor microcosm part

Each of the four hatching period × pesticide treatment combinations was replicated in twelve microcosms, giving a total of 48 microcosms. The set of twelve replicated microcosms per treatment combination consisted each time of two microcosms of each of the six populations. Hence, each microcosm contained larvae from a single population. Microcrosms were 10 L polypropylene containers filled with 3 L dechlorinated tap water and 3 L of water from an adjacent pond (filtered through a 500 µm mesh). Microcosms were placed at an outdoor experimental area in Heverlee, Belgium.

To allow the development of *Daphnia* and protozoan populations (food resource for the small damselfly larvae), the microcosms were set up ca. two weeks prior to the introduction of the larvae. We inoculated each container with ca. 300 *D. magna*. In addition, we added grass to stimulate growth of protozoa. We covered each container with a net to prevent predators entering and adult damselflies escaping.

We introduced 30 *C. puella* larvae in each container (in total 1,440 larvae), corresponding to typical field densities of coenagrionid damselfly larvae (Corbet 1999). We introduced the larvae of each period when ca. three weeks old (26-27 July 2014 for the early period and 6-7 September 2014 for the late period), thereby maintaining the ca. 40 day difference in hatching dates of the two cohorts. Throughout the outdoor microcosm experiment, we provided larvae with *ad libitum* food by adding weekly ca. 300 *D. magna* obtained from outdoor stock tanks. We reduced the feeding frequency to biweekly during periods when temperatures dropped below 10 °C, as larval growth of the study species ceases at these temperatures (Waringer and Humpesch 1984). To prevent freezing, we moved the microcosms to an unheated indoor facility for 45 days during 27 December 2014 – 12 February 2015, when outdoor temperatures dropped below 0 °C. Daily mean water temperatures when placed inside were ca. 4.3 °C (see also Fig. S1 in Appendix S2). Afterwards, microcosms were placed again outside.

Starting 4 May 2015, we applied three pesticide pulses to half of the microcosms, with three days between pulses. This simulates spring pesticide applications and the associated run-off in nearby surface waters (Dabrowski et al. 2002). The nominal esfenvalerate concentration of

0.2 µg/L induces mortality and reduces growth rate in another *Coenagrion* damselfly (Dinh Van et al. 2016). Although this concentration exceeds the predicted environmental concentrations (e.g. 0.06 µg/L for a realistic scenario; EFSA, 2014), it falls within the range of esfenvalerate concentrations detected in natural water bodies, e.g. in Denmark (up to 0.66 µg/L;Amt, 1999) and the USA (up to 0.8 µg/L; Bacey et al. 2005; Cooper et al. 2003; Werner et al. 2004). Moreover, regulatory surface water models, even when simulating most realistic scenarios, strongly underpredict measured field concentrations; especially for hydrophobic insecticides such as esfenvalerate (Knäbel et al. 2012). Importantly, our main aim was to test for a proof-of-principle for carry-over effects of pesticides and their dependence on the hatching period, rather than fine tuning the specific risk assessment of esfenvalerate. We initially prepared a $2 \times 10^5$ µg/L stock solution by dissolving 1 mg esfenvalerate powder (purity >99%, Sigma-Aldrich) in 5 mL absolute ethanol. This stock solution was further diluted with filtered pond water (mesh size: 500 µm) to obtain a spraying solution with a concentration of 24 µg/L. Fifty millilitre of this spraying solution was gently poured over the surface of the microcosms to obtain the nominal esfenvalerate concentrations of 0.2 µg/L. For the control treatment, we added 50 mL of ethanol dissolved in filtered pond water with a concentration of 24 µL/L ethanol, i.e. the ethanol concentration of the esfenvalerate treatment, to the microcosms. Growth and behavior of damselfly larvae are not affected by ethanol concentrations up to $5 \times 10^3$ µL/L (Lizanne Janssens, unpublished data). The esfenvalerate concentration in the containers 20 min after application was 0.11 µg/L, whereas after three days, just before the next pulse, the concentration was below the detection limit (< 0.04 µg/L). Concentrations were analyzed from pooled water samples from 10 microcosms, using gas chromatography in combination with mass spectrometry, with tris(1,3-dichloroisopropyl)phosphate as an internal standard, by the research laboratory Lovap NV (Geel, Belgium). No purification was needed for these analyses. We additionally measured dissolved oxygen, pH and conductivity in a subset of microcosms (see Appendix S2). Although pH levels were relatively high (9.9-10.1), damselfly larvae are generally tolerant to a wide range of pH (Corbet, 1999). Note that esfenvalerate becomes unstable in alkaline conditions (Adelsbach and Tjeerdema 2003), possibly causing a decreased bioavailability in our experiment, hence making any reported pesticide-induced responses conservative. Dissolved oxygen and conductivity values were within the recorded range for the larvae of the study species (Sternberg 1999).

### Outdoor insectary part

After the pesticide exposure period, we daily checked the microcosms for emerging adults. These were kept overnight at a nearby indoor facility to allow for hardening of the exoskeleton. The next day, we gave each individual a unique number on a hindwing using a black permanent marker (Staedtler Lumocolor, Staedtler, Germany). Next, we released them all into a single large outdoor insectary (12 × 5.5 × 2.5 m) that consisted of a metal framework covered with mosquito netting (mesh size 1 mm). The insectary contained at one end an artificial pond (65 cm diameter, 30 cm depth, 90 L) with aquatic vegetation for oviposition, and at the other end tall vegetation for foraging. Damselflies developed and behaved normally in this insectary resulting in many oviposition events (see Results; also De Block and Stoks 2005). As the insectary was placed in a field with tall grass, it contained a large diversity of prey items. In addition, we regularly released lab-cultured *Drosophila* into the insectary as additional food source.

Starting from the introduction of the first adult in the insectary, we daily checked for mating events from 9:00 until 17:00. Individuals that were copulating, ovipositing or seen in tandem (paired individuals not copulating or ovipositing) were recorded as participating in mating events (as in Thompson et al. 2011). Identities of individuals were determined either directly by eye, or by using close-focusing binoculars (Kite Optics, Belgium). The average duration of an oviposition event in the study species is 86 min, whereas pairs remain in tandem on average for 111 min (Banks and Thompson 1985). Therefore, and because pairs gathered at the small artificial pond for reproduction, we are confident that we observed all matings. As weather conditions influence reproductive activity and survival of the study species (Banks and Thompson 1987; Thompson 1990; Thompson et al. 2011), we obtained data of precipitation, hours of sunshine and temperature (see Appendix S3 for details).

### Response variables

We weighed all larvae from each microcosm on 2-3 May 2015 (just before the exposure) and again on 13-14 May 2015 (after the exposure period) to the nearest 0.01 mg using an electronic balance (AB135-S, Mettler Toledo, Columbus, OH, USA). Using mean per capita mass per microcosm, we calculated growth rate as ($\ln_{\text{final mass}} - \ln_{\text{initial mass}}$)/number of days. Survival during the pesticide exposure period was calculated as the percentage change in larval density per microcosm. Starting from the numbers of larvae that survived the pesticide exposure period, we calculated post-exposure survival as the percentage of adults that emerged alive with fully expanded wings. This measure includes mortality both during the post-exposure larval period and during metamorphosis. We defined age at emergence as the time from egg hatching to adult emergence, hence representing larval development time. Mass at emergence was quantified by weighing the one day old adults with the same electronic balance used for larva weighing.

Once in the insectary, we calculated lifetime mating success (LMS) separately for each individual as the total number of matings during its lifespan. We estimated lifespan as the number of days between emergence and the last day an animal was seen mating, hence was only calculated for animals that mated.

Note that larval growth rate and survival during the pesticide exposure period were calculated at the microcosm level (larvae were not individually marked), whereas all other endpoints are at the individual level. Starting from the adult stage, we could determine the sex of the animals. Given that the pesticide exposure did not affect the metamorphic traits, adult life history and fitness of the males (data not shown), and because effects on females are more relevant for population dynamics, we only present data of these traits for females to keep the manuscript focused.

## Statistical analyses

### *Analyses of mean effects of treatments*

To test for an effect of the pesticide treatment and hatching period on larval and adult traits and fitness, we constructed separate (generalized) linear mixed-effect models (GLMMs) per trait. Pesticide treatment (control versus pesticide) and hatching period (early versus late), as well as their interaction were included as categorical fixed effects. Except for the LMS model with a Poisson error structure and a log link function, all models had a normal error distribution and the identity link function. The model for growth rate included initial mass as a covariate to control for potential mass-dependent growth rate (Tammaru and Esperk 2007). As the number of larvae in microcosms may affect age and mass at emergence independent of the treatments, we included post-exposure larval density per microcosm as a covariate to the corresponding models. Finally, models for lifespan and LMS included weather variables averaged over the total lifespan (see Appendix S3) as covariates. All models where traits were measured at the microcosm level included population as a random effect. To take into account that individuals from the same microcosm (or population) are not independent replicates, models where traits were measured at the individual level had additionally microcosm identity (nested within population) as a random effect. One microcosm from the late period showed extreme low pre-pesticide survival (>3 standard deviations from the mean), and was excluded from all analyses. For the analysis of post-exposure survival, we identified one microcosm from the late period - control treatment combination as an outlier (>3 standard deviations from the mean) and excluded this microcosm from all analyses. To strengthen the interpretation of key results (see Results *Lifetime mating success*), we additionally calculated effect sizes estimated as Hedges' *d* with 95% confidence intervals (Jackson et al. 2016).

### *Path analyses*

We used structural equation modelling (SEM; Grace, 2006) to test whether any potential effect of the pesticide treatment on LMS was operating directly, or was indirectly mediated via the metamorphic traits (age and mass at emergence). We specifically applied the path analysis approach, which is a SEM method that deals explicitly with observed variables. Our data included a non-normally distributed response variable (i.e. Poisson-distributed LMS), and was hierarchically structured (i.e. nested random effects). Therefore, we applied the piecewise SEM (or 'generalized multilevel path analysis') approach, which allows translating a path diagram into a set of GLMMs (Lefcheck 2016; Shipley 2009).

To specifically evaluate the role of direct and indirect effects of the pesticide, we built three candidate models: (1) the pesticide affects fitness only directly; (2) the pesticide affects fitness only indirectly via the metamorphic traits; and (3) the pesticide affects fitness both directly and indirectly (Fig. 1). As the pesticide effect on LMS was modulated by period (see results of GLMMs), we ran separate path models for early and late females with the pesticide treatment as the predictor variable. All models included paths going from age and mass at emergence to LMS. Further, to correct for potential density-mediated effects, we included paths going from larval density per microcosm to age and mass at emergence (results not shown). As the effects on LMS remained when the weather variables were removed from the GLMMs (data not shown), we did not include them in the path analysis. We included correlated errors between age and mass at emergence to capture the potential trade-off between both variables. The error structure and random effects of the GLMMs used to build the path models were identical to the ones used in the mean analyses (see above). For all path models, the categorical variable 'pesticide treatment' was binary coded: control = 0, pesticide = 1. Hence, a negative path going from pesticide treatment to LMS, for example, should be interpreted as decreased LMS for pesticide-exposed females.

We selected the most parsimonious among the three candidate path models using the AICc (Akaike Information Criterion, corrected for small sample size) implemented for path models (Shipley 2013). For this, we calculated and compared ΔAICc scores and relative support for each model (AICc weights), where lower AICc scores and higher AICc weights indicate better models (Burnham and Anderson 2002). The overall fit of path models was evaluated with Shipley's test of d-separation (Shipley 2009), which tests for missing paths in the model. Using the combined significance of these unrealized paths, a Fisher's *C* statistic is calculated and compared to a $\chi^2$ distribution to reject ($P < 0.05$) or accept ($P > 0.05$) the model.

All analyses were performed using R version 3.3.2 (R Development Core Team 2015) We used the 'lme4' (Bates et al. 2015) and 'nlme' (Pinheiro et al. 2016) packages for GLMMs, the 'car' package (Fox and Weisberg 2011) for testing fixed effects with Wald- $\chi^2$, the 'piecewiseSEM' package (Lefcheck 2016) for conducting path analyses, the 'MuMIn' package (Barton 2015) to calculate AICc weights, and the 'effsize' package (Torchiano 2017) to estimate Hedges' *d*. We report standardized coefficients for the path models. Mass at emergence and lifespan were log-transformed to meet model assumptions.

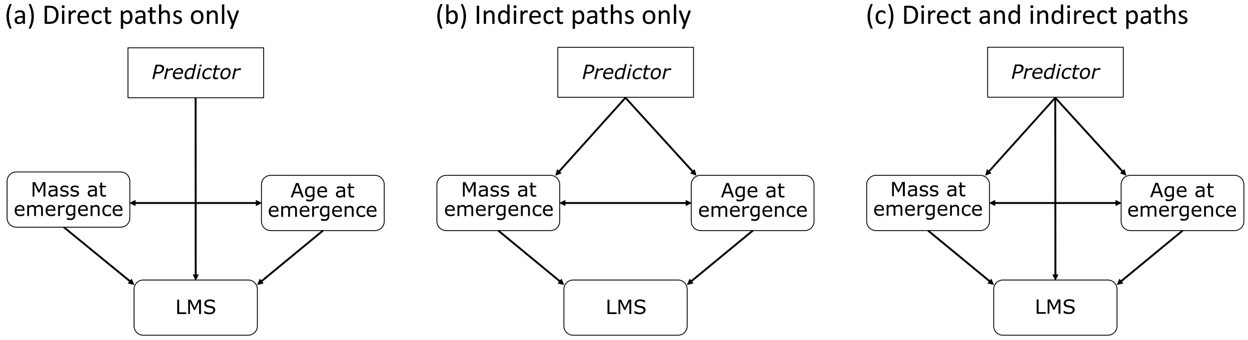

**Figure 1.** Candidate path models used to test whether the effect of a predictor on lifetime mating success (LMS) of the damselfly *Coenagrion puella* is (a) direct, (b) indirect, or (c) both direct and indirect. The predictor in the models was selected based on linear mixed effect analyses of treatment means (see main text for details). Double-headed arrows are partial correlations between traits.

## Results and discussion

Below we report effects of pesticide exposure and hatching period on life-history traits. For full details of the statistical analyses, including results for covariates, see Appendix S4.

### *Larval life history and metamorphic traits*

Larval survival prior to pesticide exposure was ca. 79% and independent of hatching period. During the pesticide exposure period, survival was reduced from ca. 89% (of the 79% pre-exposure survival) in the control group to ca. 81% in the pesticide-exposed group ($\chi_1^2 = 11.60$, $P = 0.001$, Fig. 2a). Hatching period ($\chi_1^2 = 2.87$, $P = 0.090$) and its interaction with pesticide treatment ($\chi_1^2 = 0.43$, $P = 0.514$) did not influence survival during the pesticide exposure period. Pesticide exposure reduced larval growth ($\chi_1^2 = 21.15$, $P < 0.001$, Fig. 2b). Larvae from the late period ('late larvae') had faster growth rates during the exposure period than larvae from the early period ('early larvae') ($\chi_1^2 = 6.73$, $P = 0.009$, Fig. 2b), and this did not depend on the pesticide treatment (pesticide × period: $\chi_1^2 = 0.11$, $P = 0.739$). Pesticide exposure negatively affected post-exposure survival ($\chi_1^2 = 8.37$, $P = 0.004$, Fig. 2c). Likewise, late larvae had a lower survival during the post-exposure period than early larvae ($\chi_1^2 = 12.22$, $P < 0.001$, Fig. 2c). The interaction of pesticide treatment and hatching period did not influence post-exposure survival ($\chi_1^2 = 1.55$, $P = 0.212$).

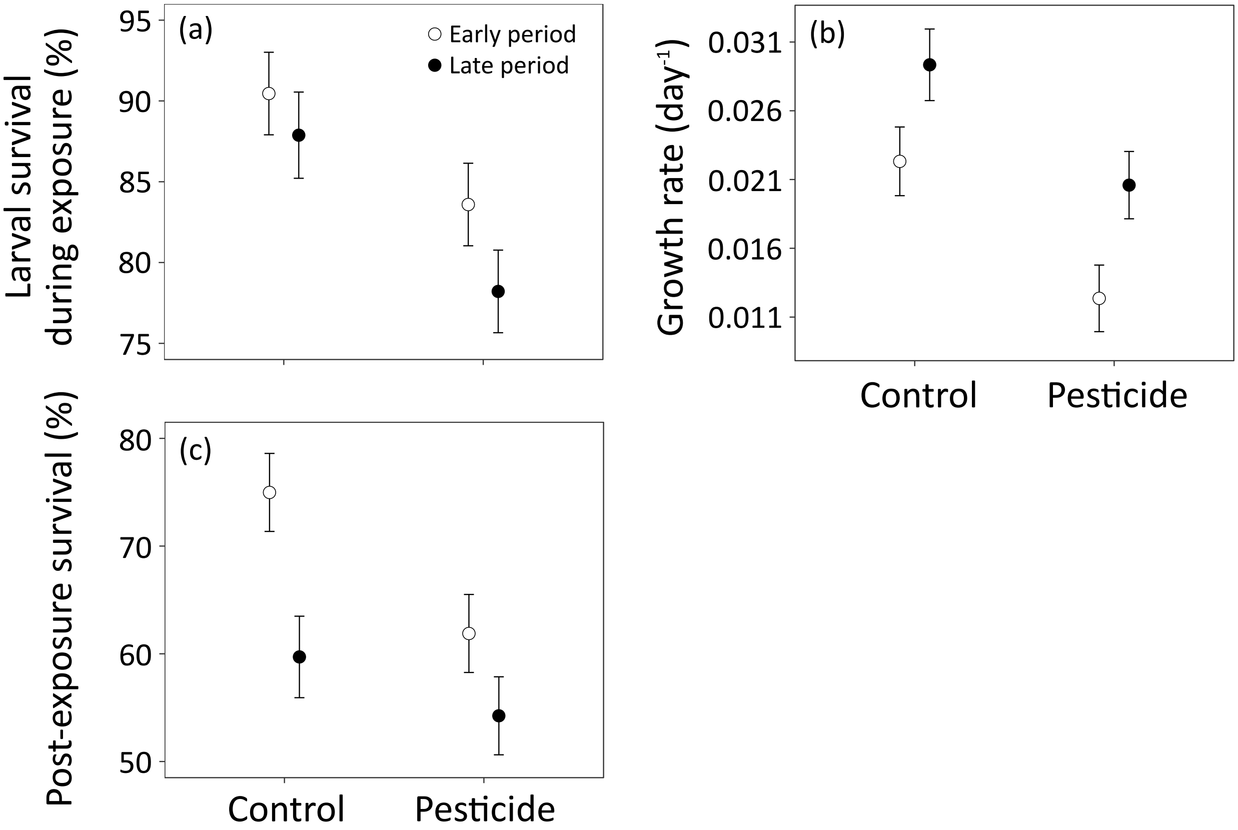

**Figure 2.** Larval survival (a), growth rate (b), and post-pesticide exposure survival (c) of the damselfly *Coenagrion puella* as a function of pesticide treatment and hatching period. Growth rate is controlled for initial mass. Note that larval survival during exposure (a) was calculated based on larvae that survived the winter (79%, see Results). Given are least square means ± 1 SE.

Age at emergence strongly differed between individuals of the two hatching periods ($\chi_1^2 = 172.25$, $P < 0.001$), with late females developing ca. 28 days faster compared early females (Fig. 3a). Pesticide exposure ($\chi_1^2 = 1.17$, $P = 0.279$) and its interaction with hatching period ($\chi_1^2 = 0.34$, $P = 0.560$) did not influence age at emergence. Damselflies from the pesticide treatment and late hatching group emerged with a slightly lower mass compared to the control and early group, respectively, yet these effects were marginally non-significant (pesticide treatment: $\chi_1^2 = 3.43$, $P = 0.064$; hatching period: $\chi_1^2 = 3.12$, $P = 0.077$, Fig. 3b). The interaction of pesticide treatment and hatching period did not influence mass at emergence ($\chi_1^2 = 0.27$, $P = 0.600$).

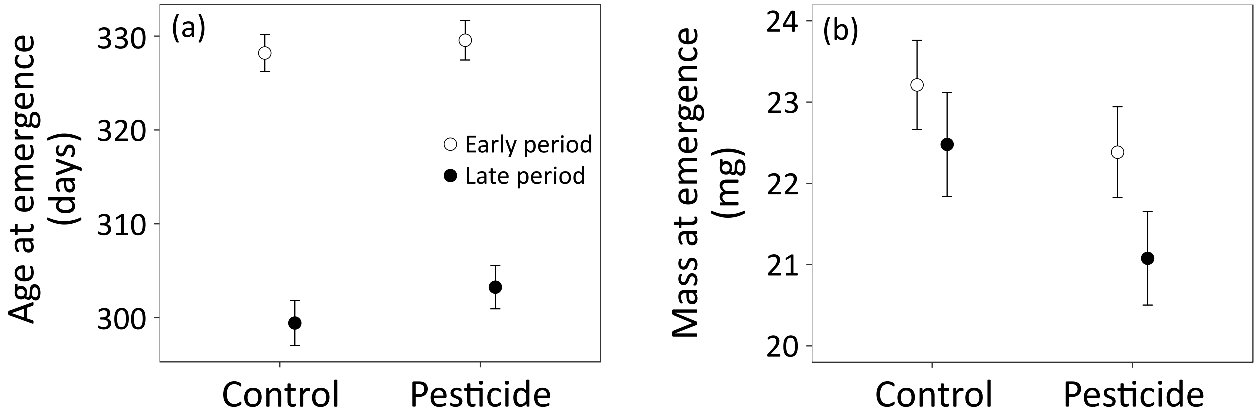

**Figure 3.** Metamorphic traits (age at emergence (a) and mass at emergence (b)) for the females of the damselfly *Coenagrion puella* as a function of pesticide treatment and hatching period. Both traits are controlled for larval density in the microcosms. Given are least square means ± 1 SE.

As expected, the chosen dose of esfenvalerate reduced survival both during and after the exposure period. This matches studies at similar esfenvalerate concentrations in aquatic insects (Beketov and Liess 2005; Kunce et al. 2015; Rasmussen et al. 2017; Rodrigues et al. 2015), including damselflies (Dinh Van et al. 2016; Janssens et al. 2017). Likewise, the pesticide reduced the growth rate, possibly due to energy re-allocation to detoxification (Rodrigues et al. 2015).

As predicted by life history theory on time constraints (Abrams et al. 1996; Rowe and Ludwig 1991), late-hatched larvae developed and grew faster than early-hatched larvae. There is ample empirical support for this pattern from a wide range of taxa (Gotthard 2001), particularly from studies with anurans (Laugen et al. 2003; Orizaola et al. 2016), and damselflies (De Block and Stoks 2004; Lowe et al. 2009). This accelerated life history decreased post-exposure survival. Similarly, survival until emergence was lower in late compared to early larvae in the damselfly *Lestes sponsa* (Dańko et al. 2017), and may reflect the trade-off between growth and survival (Lancaster et al. 2017).

### *Lifetime mating success*

Of the adults released in the insectary, 68 of the 248 females mated at least once. Pesticide exposure and hatching period did not affect lifespan (see Appendix S5). While the main effects of pesticide ($\chi_1^2 = 0.15$, $P = 0.707$) and period ($\chi_1^2 = 0.05$, $P = 0.819$) were not significant, female LMS was modulated by a significant pesticide × period interaction ($\chi_1^2 = 4.38$, $P = 0.036$, Fig. 4a,b):

previous pesticide exposure reduced LMS of early females with ca. 60% (Hedges' *d* = -0.391, 95% CI: [-0.725, -0.058]), while, if anything, the pesticide seemed to have a positive effect on LMS of late females (Hedges' *d* = 0.235, 95% CI: [-0.158, 0.627]). This drastic negative pesticide effect on female LMS was not mediated via the metamorphic traits (the 'direct paths only' model was the best supported model, Table S6 in Appendix S6). Indeed, the model for early females revealed that the pesticide exposure had a direct negative effect on LMS (as indicated by the significant negative path coefficient, Fig. 4c, Table S7). Further, increasing mass and age at emergence resulted in a higher LMS. The model for late females confirmed the pesticide had no significant effect on their LMS (Fig. 4d, Table S7).

A key finding was that the negative effects of larval pesticide exposure carried over to the adult stage and decreased LMS in early females. This result adds to the mounting evidence of carry-over effects and their impact on fitness (Harrison et al. 2011; O'Connor et al. 2014). This is in line with effects of esfenvalerate often being detected long after the exposure period ends (Forbes and Cold 2005; Liess and Schulz 1996; Palmquist et al. 2008; Rasmussen et al. 2017), and being able to bridge metamorphosis (Dinh Van et al. 2016; Schulz and Liess 2001; Śniegula et al. 2017; but see Forbes and Cold 2005). The path analysis indicated that this effect was not mediated via pesticide effects on mass or age at emergence. This is in contrast with the assumption that reduction in fitness due to exposure to a stressor should be operating by affecting these two key metamorphic traits (Abrams et al. 1996; Rowe and Ludwig 1991). Although the mechanisms underlying latent effects of pesticides are not fully understood, pesticide-mediated reductions in adult energy reserves (Palmquist et al. 2008; Rasmussen et al. 2017) may be responsible. We hypothesize this 'hidden' carry-over effect on female fitness is mediated by negative effects of esfenvalerate on fat and flight muscle mass, two flight-related traits that are impaired by this pesticide in damselflies (Dinh Van et al. 2016; Śniegula et al. 2017), thereby reducing the flight performance. Similarly, studies documenting negative effects of contaminants on mating success, for example in fish (Hued et al. 2012), birds (Frederick and Jayasena 2011), bed bugs (Crawley et al. 2017), and moths (Wei et al. 2004) suggest impaired reproductive behavior as the underlying mechanism. Compared to early females, the LMS of late females did not seem to be as strongly influenced by the pesticide; possibly because more

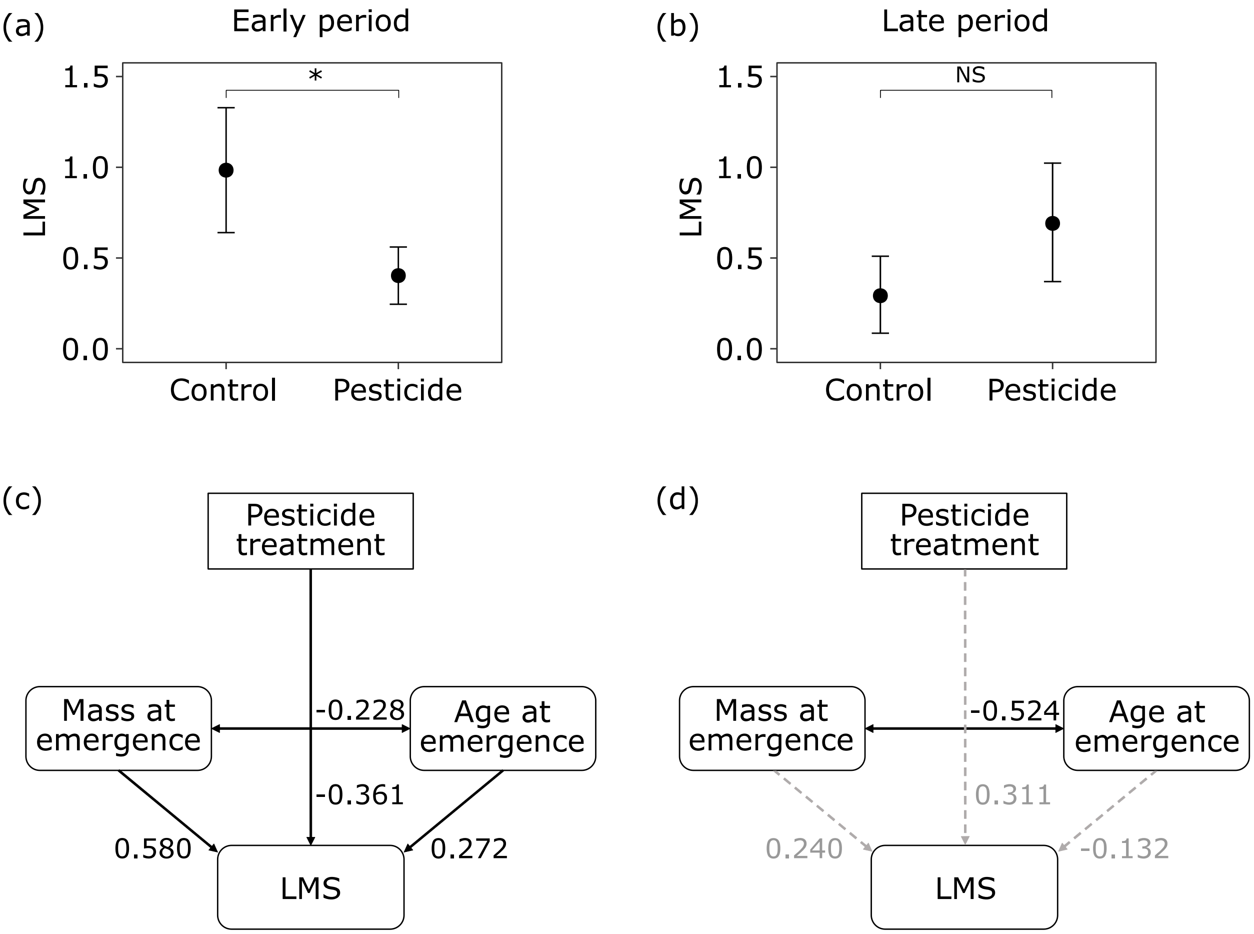

**Figure 4.** Lifetime mating success (LMS) as a function of pesticide treatment (a,b) and path diagrams depicting the effects of the pesticide treatment on life-history traits and LMS (c,d) for the females of the damselfly *Coenagrion puella*. Separately shown are plots and path diagrams for the early hatching period (a,c) and late hatching period (b,d). Given are least square means ± 1 SE for the upper plots. Pesticide treatment was significant in the early period (*), but not in the late period (NS). Dashed grey lines indicate non-significant paths, whereas double-headed arrows are partial correlations between traits. Binary coding for pesticide treatment in the path model: control = 0, pesticide = 1. Standardized path coefficients are given next to the arrows (see Table S6 for details).

vulnerable late larvae were already eliminated during metamorphosis (i.e. lower post-exposure survival in the late period). The apparent positive effect of the pesticide exposure on late period animals (Fig. 4b) may be a hormetic response, not uncommon in ecotoxicology (Cedergreen et al. 2005), operating via a mechanism not identified in the present study.

### *General implications for risk assessment of pesticides*

Using a powerful longitudinal design that integrated an exposure part in microcosms with the monitoring of lifetime fitness in a large outdoor insectary, our study provides the first data on carry-over effects of a pesticide across metamorphosis on adult lifetime fitness under seminatural conditions. Although the here used concentration of esfenvalerate (0.11 µg/L) is ca. two times higher than the predicted environmental concentration based on worst-case scenarios (EFSA 2014), regulatory surface water models simulating realistic scenarios strongly underpredict actual measured field concentrations; especially for hydrophobic insecticides such as esfenvalerate (Knäbel et al. 2012). This is in agreement with several studies reporting substantially higher (up to 0.8 µg/L) esfenvalerate concentrations in natural water bodies (Amt 1999; Bacey et al. 2005; Cooper et al. 2003; Werner et al. 2004). Nevertheless, our aim was to test under seminatural conditions for mechanisms possibly responsible for the failure of current risk assessment of pesticides in general (detailed below), rather than fine tuning regulatory limits for esfenvalerate.

Aquatic habitats are usually dominated by insects with complex life cycles, i.e. with aquatic larvae metamorphosing into terrestrial adults. Although current water quality standards acknowledge latent effects of pesticide exposure (Duncan et al. 2009), they do not explicitly take into account carry-over effects across metamorphosis, hence assume that protection of aquatic stages translate to the protection of adults. We provide a proof-of-principle that this assumption is not met, and suggest that the current guidelines would have erroneously concluded the pesticide effect to be weak if looking only at responses at the larval stage (for metals: Debecker et al. 2017; Schmidt et al. 2013). Notably, a simple simulation based on our results and the general relationships between fitness components and the number of offspring that reaches adulthood (based on studies in a natural *C. puella* population: Banks and Thompson 1987; Thompson et al. 2011) revealed that when including the carry-over effects, the pesticide treatment is expected to reduce the number of offspring reaching maturity by more than three times (see Appendix S7 for details of the simulation). Importantly, this simulation shows that the pesticide-induced mortality in the larval stage accounted only for ca. 24% of the total impact on the population in the next generation, while the rest of the impact was captured by variation in LMS (Appendix S7). These delayed effects after metamorphosis could not be predicted by effects on the two key metamorphic traits (age and mass at metamorphosis), thereby violating the assumption of life history theory that metamorphic traits should predict the effects of stressors on adult fitness (Roff 2002). This further complements the few studies reporting that post-metamorphic traits are weak predictors of carry-over effects of ecological stressors on adult fitness (De Block and Stoks 2005; Earl and Whiteman 2015; Van Allen et al. 2010). This argues against using mass and age at metamorphosis as proxies for effects on adult fitness in ecotoxicological studies (Rohr and Palmer 2005; Goedkoop et al. 2010; Janssens and Stoks 2013), and underscores the importance of identifying the underlying mechanisms of carry-over effects (O'Connor et al. 2014; Debecker et al. 2015).

We further identified an overlooked, yet important temporal component associated with time constraints strongly determining the impact of pesticide exposure. Indeed, the larval pesticide exposure affected lifetime fitness only in early-hatched females, and not in the more time-stressed late-hatched females. This large impact of hatching phenology is striking because pesticide exposure occurred ca. 10 months after egg hatching, thereby simulating the general scenario where aquatic insects that hatched in summer are exposed the next spring to pesticide pulses. Given the widespread variation in hatching periods in natural populations and the associated differences in time constraints (De Block and Stok 2005; Orizaola et al. 2013), this is expected to be a general factor that may critically affect the consequences of pollutant exposure on adult fitness. Ecotoxicology tests on field-collected animals (as frequently done in mesocosm tests) typically ignore the hatching date. Hence, the here identified mechanisms may introduce considerable noise when animals differing in hatching period are being tested, and may explain differences between toxicological studies; even when using animals from the same study population.

## Acknowledgments

We thank Selina Müller for her dedicated help during the experiment, and Geert Neyens for setting up the insectary. Comments from four anonymous reviewers considerably improved our manuscript. Financial support came from the Belspo project SPEEDY (IAP- project P7/04) and research grants from the KU Leuven (PF/2010/07 and C16/17/002) and the FWO research network EVENET.

## Appendix S1

**Table S1.** Results of the linear mixed effect models testing for the effects of pesticide treatment, hatching period, and urbanisation level on age at emergence and mass at emergence for the females of the damselfly *Coenagrion puella*.

| | Age at emergence | | Mass at emergence | |
|---|---|---|---|---|
| | $\chi^2$ | p | $\chi^2$ | p |
| Pesticide treatment (Pesticide) | 1.13 | 0.288 | 2.61 | 0.106 |
| Hatching period (Period) | **178.03** | **< 0.001** | **4.73** | **0.030** |
| Urbanisation level (Urban) | 1.03 | 0.311 | 0.58 | 0.446 |
| Pesticide × Period | 0.20 | 0.657 | 0.06 | 0.804 |
| Pesticide × Urban | 0.53 | 0.466 | 1.74 | 0.187 |
| Period × Urban | 0.03 | 0.860 | 2.26 | 0.133 |
| Pesticide × Period × Urban | 0.28 | 0.597 | 0.01 | 0.936 |
| Larval density in microcosm | **9.52** | **0.002** | **35.22** | **< 0.001** |

**Table S2.** Results of the (generalized) linear mixed effect models testing for the effects of pesticide treatment, hatching period, and urbanisation level on lifespan and lifetime mating success for the females of the damselfly *Coenagrion puella*.

| | Lifespan | | Lifetime mating success | |
|---|---|---|---|---|
| | $\chi^2$ | p | $\chi^2$ | p |
| Pesticide treatment (Pesticide) | 2.07 | 0.150 | 0.07 | 0.785 |
| Hatching period (Period) | 0.31 | 0.579 | 0.21 | 0.645 |
| Urbanisation level (Urban) | 0.87 | 0.351 | 0.46 | 0.499 |
| Pesticide × Period | 0.67 | 0.412 | **4.81** | **0.028** |
| Pesticide × Urban | 0.03 | 0.854 | 0.28 | 0.596 |
| Period × Urban | 3.44 | 0.064 | 1.14 | 0.286 |
| Pesticide × Period × Urban | 1.90 | 0.168 | 2.19 | 0.139 |
| Temperature | **20.84** | **< 0.001** | **5.00** | **0.025** |
| Precipitation | 0.01 | 0.904 | **30.80** | **< 0.001** |
| Sunshine | **5.06** | **0.024** | **7.20** | **0.007** |

## Appendix S2 – Abiotic parameters

Dissolved oxygen and pH were measured in the microcosms before, during, and after the pesticide application. Due to logistic reasons, conductivity could only be measured after the pesticide exposure. We installed temperature loggers (HOBO UTBI-001, Onset Computer Corporation) in two randomly selected microcosms on 22 December 2014, and recorded water temperatures throughout the experiment (at 15-min intervals). Abiotic measurements except temperature were taken from a subsample of eight microcosms, with four microcosms per pesticide treatment (control and pesticide exposure). As temperature is not expected to differ between treatments, we present the average temperature profile of the two microcosms (Fig. S1). Dissolved oxygen (mg/L), conductivity (µS), and pH were measured with a portable multimeter (HQ40d, Hach, Loveland, Co, USA). Measurements expect for temperature were carried out around noon (between 11 am and 3 pm) in the centre of the microcosm at a depth of ca. 10 cm where most of damselfly larvae were present. Note that microcosms did not contain air stones for additional aeration, the high values of dissolved oxygen in microcosms (see below) indicated that this was not needed.

To test whether abiotic parameters in the containers differed between the pesticide treatments, we ran repeated-measures ANOVAs with pesticide treatment as fixed effect, and the days of measurements as repeats.

Dissolved oxygen did not differ significantly between the control (mean ± 1 SE: 14.29 ± 0.69 mg/L) and pesticide treatment group (15.88 ± 0.88) ($F_{1,59}$=1.73, $P$ = 0.193; Fig. S2a). Similarly, conductivity values were similar between the control (219.29 ± 6.77 µS) and pesticide treatment group (231.04 ± 9.62) ($F_{1,59}$=0.22, $P$ = 0.645; Fig. S2c). Pesticide-treated microcosms had a slightly higher pH (10.10 ± 0.09) compared to the control microcosms (9.91 ± 0.05) (Fig S2b, $F_{1,59}$=4.04, $P$ =0.0489).

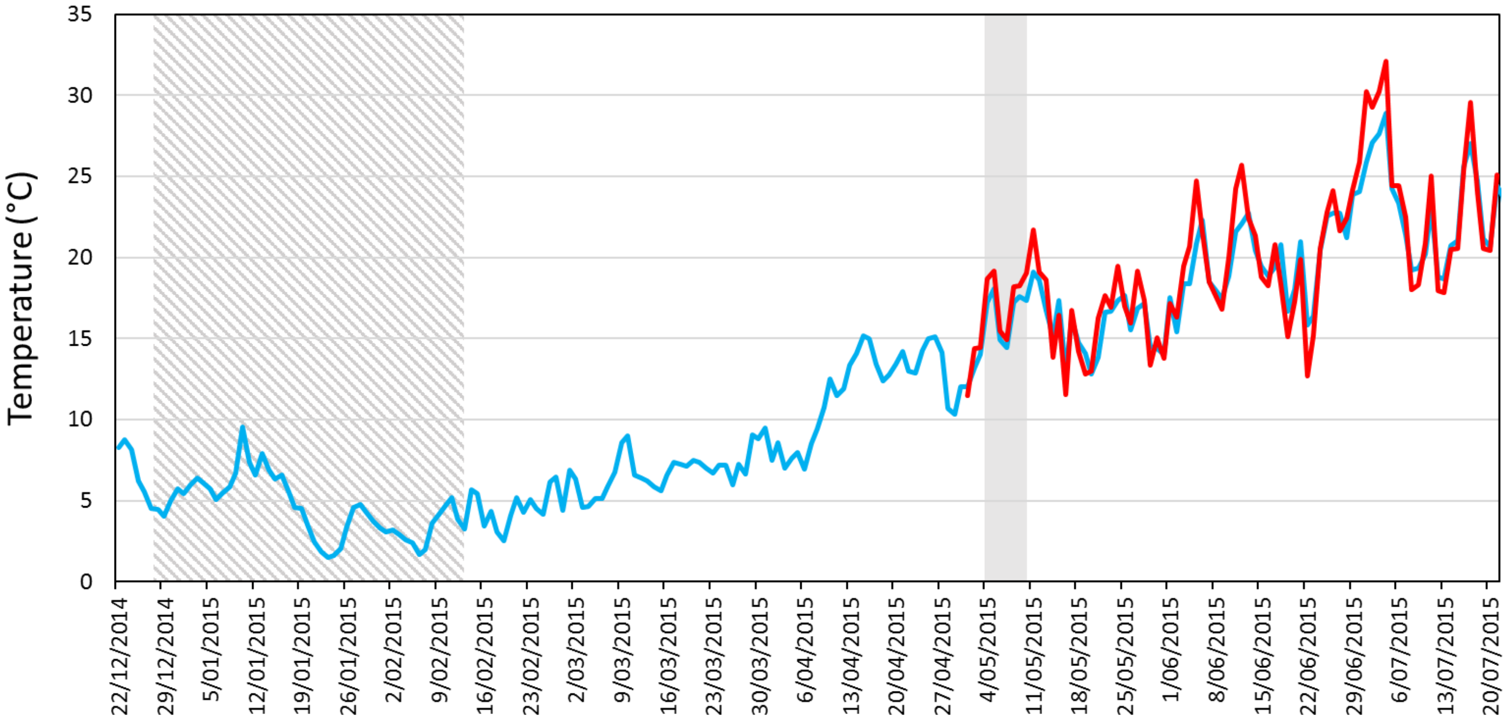

**Figure S1.** Mean daily water temperatures in the microcosms (blue line) and air temperature in the insectary (red line) during the experiment. The dashed area indicates the period when microcosms were placed temporarily indoors, whereas the grey area represents the pesticide exposure period.

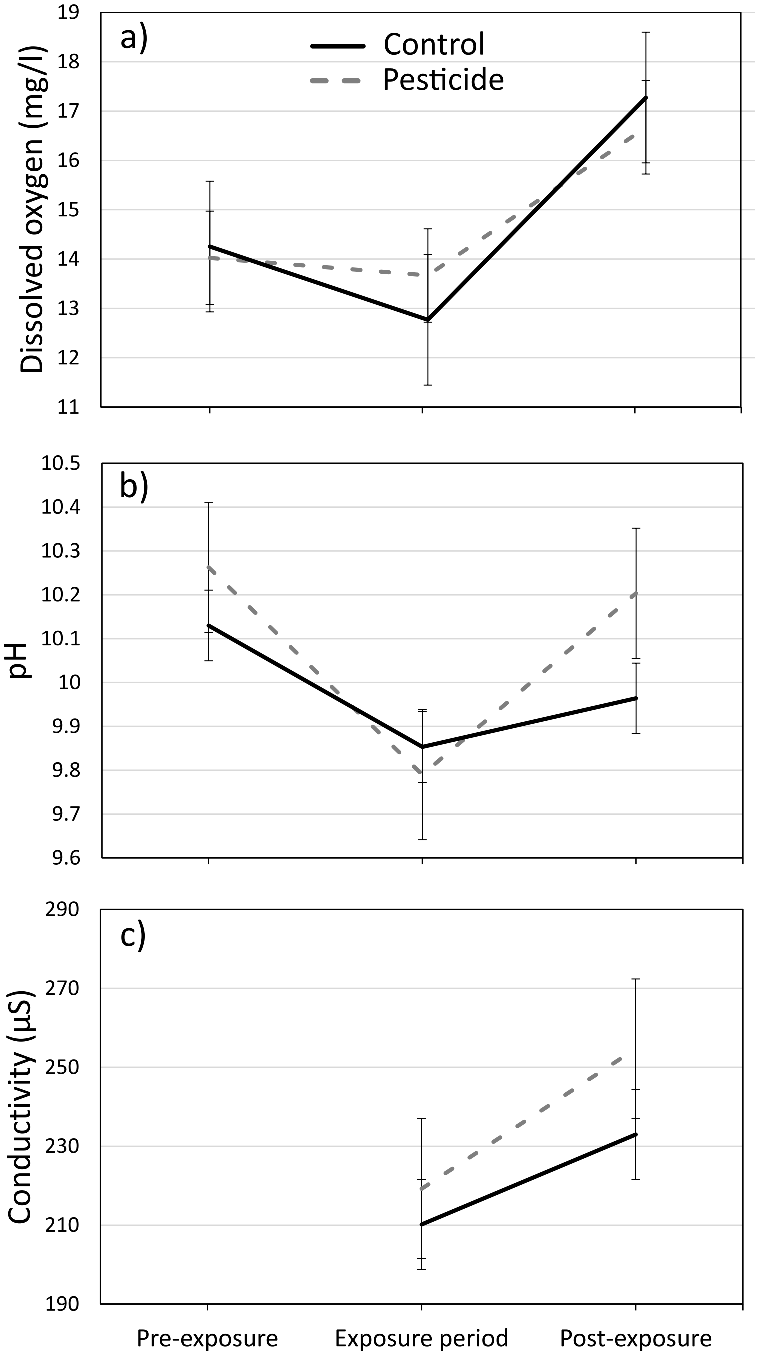

**Figure S3.** Means of dissolved oxygen (a), pH (b), and conductivity (c) in the microcosms as a function of the pesticide treatment. Note that conductivity could be measured only during exposure and post-exposure period. To aid visual interpretation, we pooled values of each parameters per measurement stage (pre-exposure, exposure and post-exposure periods). Given are means (±1 SE) across microcosms.

## Appendix S3 - Weather variables

As weather conditions are known to influence reproductive activity and survival of the study species (Banks and Thompson 1987; Thompson 1990; Thompson et al. 2011), we obtained data of total daily precipitation (mm) and total daily sunshine (h) from the Royal Meteorological Institute of Belgium, measured by three nearby weather stations (max. distance to experimental area was 6.4 km). Weather variables were averaged over the measurements from the three weather stations. Mean daily temperature (°C) was measured using a data logger (HOBO UTBI-001, Onset Computer Corporation) installed in the outdoor insectary (see Fig. S1). Per individual that mated at least once, we calculated the means of each of the three weather variables averaged over its lifespan (i.e. number of days between emergence and the last day it was seen mating). For animals that never mated, we calculated only the mean over the maturation period, starting from the day of release in the insectary until the average day of first reproduction of all females that mated at least once.

## Appendix S4 – Results of (generalized) linear mixed effect models

**Table S3.** Results of the linear mixed effect models testing for the effects of pesticide treatment and hatching period on larval survival, growth rate and post-exposure survival of the damselfly *Coenagrion puella*.

| | Larval survival | | Growth rate | | Post-exposure survival | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Pesticide treatment (Pesticide) | **11.598** | **0.001** | **21.146** | **<0.001** | **8.367** | **0.004** |
| Hatching period (Period) | 2.874 | 0.090 | **6.729** | **0.009** | **12.220** | **<0.001** |
| Pesticide × Period | 0.426 | 0.514 | 0.111 | 0.739 | 1.555 | 0.212 |
| Mass before exposure | | | **41.440** | **<0.001** | | |

Significant effects ($P < 0.05$) are indicated in bold.

**Table S4.** Results of the (generalized) linear mixed effect models testing for the effects of pesticide treatment and hatching period on age at emergence, mass at emergence, and lifetime mating success for the females of the damselfly *Coenagrion puella*.

| | Age at emergence | | Mass at emergence | | Lifetime mating success | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Pesticide treatment (Pesticide) | 1.172 | 0.279 | 3.431 | 0.064 | 0.147 | 0.707 |
| Hatching period (Period) | **172.247** | **<0.001** | 3.122 | 0.077 | 0.052 | 0.819 |
| Pesticide × Period | 0.339 | 0.560 | 0.274 | 0.600 | **4.376** | **0.036** |
| Larval density in microcosm | **9.968** | **0.002** | **34.586** | **<0.001** | | |
| Temperature | | | | | **5.556** | **0.018** |
| Precipitation | | | | | **31.954** | **<0.001** |
| Sunshine | | | | | **6.821** | **0.009** |

Significant effects ($P < 0.05$) are indicated in bold.

## Appendix S5 – Results for lifespan

On average, mated females lived ca. 18.5 days (Fig. S4). None of the treatments influenced female lifespan (Table S5).

**Table S5.** Results of the linear mixed effect model testing for the effects of pesticide treatment and hatching period on lifespan for the females of the damselfly *Coenagrion puella*.

| | Lifespan | |
|---|---|---|
| | $\chi^2$ | *P* |
| Pesticide treatment (Pesticide) | 1.338 | 0.247 |
| Hatching period (Period) | 0.293 | 0.588 |
| Pesticide × Period | 0.492 | 0.483 |
| Temperature | **22.299** | **<0.001** |
| Precipitation | **0.029** | **0.866** |
| Sunshine | 4.814 | 0.028 |

Significant effects ($P < 0.05$) are indicated in bold.

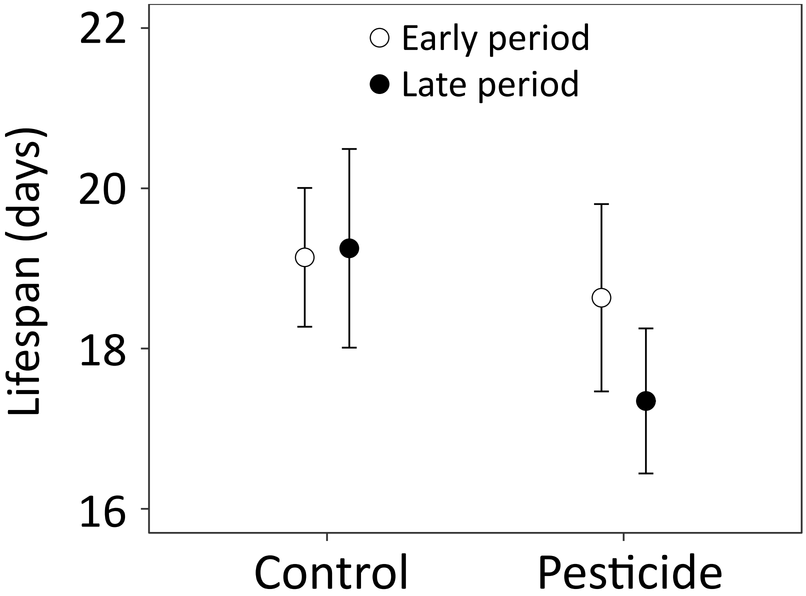

**Figure S4.** Lifespan of the females of the damselfly *Coenagrion puella* as a function of pesticide treatment and hatching period. Given are least square means ± 1 SE, controlled for weather conditions.

## Appendix S6 – Model fits and path coefficients of path analyses

**Table S6.** Parameters for model fits and model selection, with pesticide treatment as predictor.

| Candidate path models | Fisher's *C* | d.f. | *P* | AICc | ΔAICc | ωAICc |
|---|---|---|---|---|---|---|
| *Hatching period: Early* | | | | | | |
| Only direct paths | **2.56** | **6** | **0.862** | **38.81** | **0** | **0.793** |
| Only indirect paths | 5.96 | 4 | 0.202 | 44.78 | 5.97 | 0.040 |
| Direct + indirect paths | 0.50 | 2 | 0.780 | 41.93 | 3.12 | 0.167 |
| *Hatching period: Late* | | | | | | |
| Only direct paths | **5.07** | **6** | **0.535** | **40.59** | **0** | **0.726** |
| Only indirect paths | 6.63 | 4 | 0.157 | 44.96 | 4.37 | 0.082 |
| Direct + indirect paths | 2.04 | 2 | 0.360 | 43.24 | 2.65 | 0.193 |

Parameters are given separately for only the early hatched and only the late hatched group. Best supported models per period are indicated in bold. AICc = Akaike's Information Criterion corrected for sample size, ΔAICc = difference in AICc between best model and other candidate models, ωAICc = Akaike weights, representing relative support for model.

**Table S7.** Standardized path coefficients for the best supported path models explaining lifetime mating success of females damselflies.

| | | Hatching period: Early | | | Hatching period: Late | | |
|---|---|---|---|---|---|---|---|
| Response | Predictor | Coefficient | SE | *P* | Coefficient | SE | *P* |
| Age at emergence | Larval density in microcosm | 0.415 | 0.118 | 0.003 | 0.057 | 0.104 | 0.590 |
| Mass at emergence | Larval density in microcosm | -0.586 | 0.097 | < 0.001 | -0.335 | 0.121 | 0.014 |
| Lifetime mating success | Pesticide treatment | -0.361 | 0.002 | < 0.001 | 0.311 | 0.272 | 0.253 |
| Lifetime mating success | Mass at emergence | 0.580 | 0.002 | < 0.001 | 0.240 | 0.159 | 0.130 |
| Lifetime mating success | Age at emergence | 0.272 | 0.002 | < 0.001 | -0.132 | 0.131 | 0.315 |
| Age at emergence | Mass at emergence | -0.228 | | < 0.001 | -0.524 | | < 0.001 |

Path coefficients are given separately for the models including only early and only late hatched individuals. Note that the last row represents partial correlation between age and mass at emergence.

## Appendix S7 – Simulation to estimate pesticide effect at the population level

To evaluate the consequences of the pesticide exposure at the population level in the early females, we simulated the effect of the pesticide based on (i) the results reported in the present study, and (ii) the general relationships between fitness components and the number of offspring that reaches adulthood in *C. puella* (based on studies in a natural population: Thompson et al. 2011; Banks and Thompson 1985b). Specifically, we calculated the number of total mating events of a hypothetical population starting with 100 females in the egg stage based on the results of the present study. Next, we extrapolated the number of mating events into the number of resulting female offspring reaching adulthood using estimations for number of eggs produced (based on Banks and Thompson 1985b) and egg-to-adult survival rate (based on Thompson et al. 2011). The simulation revealed 82 female offspring reaching adulthood the next generation in the control group, whereas the next generation of the pesticide treated group consisted of only 27 females (Table S8, see below for details of the calculations). This simulation further showed that the lethal effect of the pesticide in the larval stage captured only ca. 24% of the total impact on the population in the next generation, while the post-metamorphic effect is much more important (capturing ca. 76%).

**Table S8.** Results of the simulation for the control and pesticide exposure scenarios. Both treatment groups start with a hypothetical 100 females.

| Treatment | Number of survivors to adulthood | Total number of matings | Total number of eggs | Number of offspring surviving to adulthood |
|---|---|---|---|---|
| Control | 67 | 65 | 11761 | 82 |
| Pesticide | 52 | 21 | 3894 | 27 |

*Number of females surviving to adulthood* was calculated based on the empirical results of the present study; 67% for the control group, and 52% for the pesticide treated group (both from the early period) survived to adulthood. Note that these survival percentages integrate the survival percentages both during and after pesticide exposure (survival during exposure; early-control: 90%, early-pesticide: 84%; post-exposure survival; early-control: 75%, early-pesticide: 62%). *Total number of matings* was estimated by the multiplication of the number of survived females with the lifetime mating success (LMS) as quantified in our study. LMS for the control was 0.966 and for the pesticide group it was 0.399 (both from the early period). *Total number of eggs* was calculated using the equation by Banks and Thompson (1985b, p824, Fig.9) that was specifically derived from field data of the study species: Total number of eggs = 178.8 x + 139.3 (originally the ‘x’ here represents ‘number of clutches’, and we replaced this with ‘number of matings’, assuming all mating events are followed by oviposition [NT and RS, personal observation]). *Number of offspring surviving to adulthood* was estimated using the value of 0.7%, as reported in Thompson et al. (2011, p911) for a natural population of the study species.

## CHAPTER 5

# Pathways to fitness: carry-over effects of late hatching and urbanisation on lifetime mating success

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## Abstract

Life history theory and most empirical studies assume carry-over effects of larval conditions to shape adult fitness through their impact on metamorphic traits (age and mass at metamorphosis). Yet, very few formal tests of this connection across metamorphosis exist, because this entails longitudinal studies from the egg stage and requires measuring fitness in (semi)natural conditions. In a longitudinal one-year common-garden rearing experiment consisting of an outdoor microcosm part for the larval stage and a large outdoor insectary part for the adult stage, we studied the effects of two factors related to time constraints in the larval stage (egg hatching period and urbanisation) on life history traits and lifetime mating success in the males of the damselfly *Coenagrion puella*. We reared early- and late-hatched larvae from each of three rural and three urban populations from the egg stage throughout their adult life. Key findings were that both the hatching period and urbanisation shaped adult fitness, yet through different pathways. As expected, the more time-constrained late-hatched individuals accelerated their larval life history and this was associated with a lower lifetime mating success. A path analysis revealed this carry-over effect was mediated by the changes in the two metamorphic traits (reduced age and lower mass at emergence). Notably, urban males had a 50% lower lifetime mating success, which was not mediated by age and mass at emergence, and possibly driven by their shorter lifespan. Our results point to long-term carry-over effects of the usually ignored natural variation in egg hatching dates, and further contribute to the limited evidence showing fitness costs of adjusting to an urban lifestyle.

## Introduction

There is general attention for carry-over effects of conditions experienced early in life, as these may shape adult fitness (Pechenik 2006; Harrison et al. 2011; Marshall and Morgan 2011; O'Connor et al. 2014) and even population dynamics (Beckerman et al. 2002). However, tracking the fitness consequences of larval conditions in the adult stage is challenging, especially as these should ideally be measured under conditions as natural as possible (Miller and Svensson 2014). This is especially true for the majority of animals that have a complex life cycle, with a larval stage where growth occurs separated by metamorphosis from an adult stage where reproduction occurs (Wilbur 1980). As a result, the majority of empirical studies on carry-over effects rely on effects on metamorphic traits as proxies for the fitness consequences in the adult stage (reviewed in Earl and Whiteman 2015). Similarly, life history theory typically assumes the effects of larval conditions to be mediated by the adult stage via two key metamorphic traits: age and mass at metamorphosis (e.g. Rowe and Ludwig 1991; Abrams et al. 1996). Yet, these metamorphic traits not always accurately predict the effects of larval conditions on adult fitness (De Block and Stoks 2005; Tigreros 2013; Earl and Whiteman 2015; Rosa and Saastamoinen 2017), hence should be used with caution as fitness proxies. A powerful statistical approach to test whether carry-over effects are mediated by these metamorphic traits is provided by path analysis, yet few studies capitalized on it (but see Debecker et al. 2015).

Time constraints associated with seasonality are a common phenomenon experienced by many animals in the larval stage. A well-known reaction to time constraints is to adopt an accelerated life style, i.e. a fast larval growth and development, to compensate for a late start (Rowe and Ludwig 1991; Abrams et al. 1996). The reaction to time constraints, being plastic or evolved, is crucial for organisms, as failing to reach a certain developmental stage on time would result in missing out on the mating season (Gotthard 2001). Aside from the risk of ending up with zero fitness due to failure to mate, effects of time constraints can carry-over from the larval life stage to the adult stage and ultimately alter fitness (Harrison et al. 2011). Yet, our understanding of the long-term fitness effects associated with larval time constraints is limited, as most studies on carry-over effects only looked at effects on metamorphic traits (e.g. Mikolajewski et al. 2015; Orizaola et al. 2016) or juvenile fitness components (e.g. Altwegg and Reyer 2003; Dahl et al. 2012).

Variation in reproductive phenology, and associated hatching phenology, is a widespread driver of differentiation in time constraints within populations. Natural variation in hatching phenology can be large with adults metamorphosing at the start and near the end of the reproductive season. Despite this major cause of variation in time constraints within populations, few studies tested the implications of this natural variation in hatching period on life history traits and adult fitness (but see Altwegg 2002; De Block and Stoks 2005; Lowe et al. 2009; Orizaola et al. 2013). Apart from natural variation in reproductive phenology, time constraints experienced by populations can also differ spatially. For example, differences in the length of the growing seasons, hence in the intensity of time constraints, drive strong variations in life history traits and fitness across latitudinal gradients (e.g. Yamahira and Conover 2002; Laugen et al. 2003; Stoks et al. 2012; Śniegula et al. 2016). Urbanisation gradients have also been associated with changing time constraints due to extending growing seasons (Somers et al. 2013; Yang et al. 2013; Zipper et al. 2016), driven by the higher temperatures in urban compared to rural areas (Gaston et al. 2010). Yet, very few studies have tested life history responses to the assumed differentiation in time constraints between rural and urban populations (but see McLean et al. 2005; Gomez and Van Dyck 2012; Tüzün et al. 2017a), and none looked directly at fitness.

We tested how time constraints associated with hatching phenology and urbanisation carried over to shape adult fitness in the damselfly *Coenagrion puella*. We did so by quantifying effects on metamorphic traits, and lifetime mating success in a longitudinal one-year experiment starting from the egg stage that integrated an outdoor microcosm part for the larval stage with a large outdoor insectary part for the adult stage. This experimental design allowed us to explicitly test i) whether natural and spatial variation in time constraints carries over and alters adult fitness, and ii) whether these carry over effects are mediated via metamorphic traits. The study species *C. puella* is common in both rural and urban areas in Europe (Goertzen and Suhling 2013), and known to react to time constraints (Mikolajewski et al. 2015) and to differ in life history between urban and rural populations (Tüzün et al. 2017a,b). Because of stronger time constraints, we predicted an accelerated life history in the late hatching group and in rural populations, resulting in a reduced age and mass at metamorphosis, and considering the associated costs of a fast life (Metcalfe and Monaghan 2003; Dmitriew 2011), a reduced lifetime mating success.

## Materials and methods

### Study species and populations

Adult *C. puella* reproduce in early summer, and eggs are oviposited in aquatic vegetation. Larvae hatch from eggs ca. 3 weeks later, and adults emerge the next spring or early summer, after ca. 10 months of larval development (Lowe et al. 2009). The reproductive biology of *C. puella* has been extensively studied (Banks and Thompson 1985b; Thompson 1990; Lowe et al. 2009; Thompson et al. 2011; Gyulavári et al. 2014; Tüzün et al. 2017b). After emergence, adults go through a ca. 15 days maturation period where they stay away from the water to forage (Banks and Thompson 1985a). After maturation, animals return to ponds for reproduction. Males display scramble competition for females and are non-territorial. Female choice behavior is thought to be not important in this species (Banks and Thompson 1985b).

We used three rural and three urban locations for sampling damselflies, all within a 45 km radius in Flanders, Belgium. We determined the urbanisation level using % built-up area, quantified with GIS data: rural ponds were surrounded by <3% built-up area, whereas urban ponds were surrounded by >15% built-up area. As a first step, we selected three rural and three urban plots of 3 x 3 km. Next, within these plots, a subplot of 200 x 200 m with the same urbanisation level was selected. This approach ensured that both the direct environment and the broader surroundings reflect the same urbanisation level (see also Tüzün et al. 2015, 2017a,b; Kaiser et al. 2016; Piano et al. 2017).

We collected eggs from each of the six populations once in the period 13 – 22 June ("early period"), and a second time in the period 23 – 31 July 2014 ("late period"). These dates match the peak (early period) and the end (late period) of the flight season in Flanders, and resulted in two cohorts differing ca. 40 days in egg hatching time. The average lifespan of adult *C. puella* is ca. 20 days (Banks and Thompson 1985a, see also Results), hence the two collected cohorts derived from parents with non-overlapping flight periods. During each period, we collected eggs from 5-10 mated females from each pond. Eggs from the early and late periods hatched during 4 – 12 July and 16 – 19 August 2014, respectively. We kept the larvae from each female together in 1.5 L plastic containers filled with dechlorinated tap water in a temperature-controlled room (20 °C, 14:10 h light:dark photoperiod). We fed the larvae *Artemia* nauplii *ad libitum* five days a week.

### General experimental procedure

The longitudinal one-year study consisted of two integrated parts. For the outdoor microcosm part (summer 2014 – spring 2015), we set up a full factorial experiment. The study originally included besides hatching period and urbanisation level an additional pesticide exposure treatment in the larval stage. Exposure to the pesticide did not affect the here measured metamorphic traits, nor did it affect adult male life history traits and fitness. Therefore, we only report the effect of hatching period and urbanisation level on males to keep the manuscript focused. Note that excluding females from our analyses does not effect the here presented results, as sexes drastically differ in how metamorphic traits are shaped by larval treatments, hence need to be analysed separately when applying path modeling. We report the effects of the pesticide treatment on female lifetime mating success in Tüzün and Stoks (2017). We nevertheless included the pesticide treatment (and its interactions) in our statistical analyses, and reported the results of the pesticide treatment in the Appendix (Tables A1 and A2). This resulted in the following design of 2 hatching periods (early and late) × 2 urbanisation levels (rural and urban, each represented by three populations) × 2 pesticide treatments (control and 0.2 µg/L esfenvalerate). In the subsequent outdoor insectary part (late spring – summer 2015), we placed the adults that emerged from the microcosms in an outdoor insectary where we monitored adult life-history traits and lifetime mating success.

### Outdoor microcosm part

We replicated each of the eight treatment combinations with six microcosms, giving a total of 48 microcosms. As microcrosms, we used 10 L polypropylene containers filled with 3 L dechlorinated tap water and 3 L of water from an adjacent pond which was filtered through a 500 µm mesh. We placed the microcosms at an outdoor experimental area in Heverlee, Belgium.

We set up the microcosms ca. two weeks prior to the introduction of the larvae, which allowed the development of protozoan populations to provide a food resource for the small damselfly larvae. In addition, we inoculated each container with ca. 300 *Daphnia magna*. We covered each microcosm with a net to prevent predators entering and adult damselflies escaping.

We introduced 30 *C. puella* larvae in each microcosm (in total 1,440 larvae). This corresponds to typical field densities of coenagrionid damselfly larvae (Corbet 1999). Larvae of each period were ca. three weeks old (26-27 July 2014 for the early period and 6-7 September 2014 for the late period) when placed into the microcosms, hence maintaining the ca. 40 day difference in hatching phenology of the two cohorts. We provided larvae with *ad libitum* food throughout the outdoor microcosm experiment by adding weekly ca. 300 *D. magna* obtained from outdoor stock tanks. Feeding frequency was reduced to biweekly during periods when temperatures dropped below 10 °C, as larval growth of the study species ceases at these temperatures (Waringer and Humpesch 1984). To prevent freezing, we moved the microcosms to an unheated indoor facility for 45 days during 27 December 2014 – 12 February 2015, when outdoor temperatures dropped below 0 °C. During this period, daily mean water temperatures were ca. 4.3 °C. Afterwards, microcosms were placed again outside.

Starting 4 May 2015, we applied three pesticide pulses to half of the microcosms, with three days between pulses.

### Outdoor insectary part

After the pesticide exposure period, we daily checked the microcosms for emerging adults. Newly emerged individuals were kept overnight at a nearby indoor facility, allowing for the hardening of the exoskeleton. The next day, each individual was given a unique number on a hindwing using a black permanent marker (Staedtler Lumocolor, Staedtler, Germany). Marked adults were released into a large outdoor insectary (12 × 5.5 × 2.5 m) with a metal framework and mosquito netting (mesh size 1 mm). The insectary contained at one end tall vegetation for foraging, and at the other end an artificial pond (65 cm diameter, 30 cm depth, 90 L) with aquatic vegetation for oviposition. Damselflies developed and behaved normally in this insectary, evident from the many mating events (see Results; also De Block and Stoks 2005). Throughout the experimental period, adult damselflies had access to naturally occurring prey in the insectary, and we regularly released lab-cultured *Drosophila* into the insectary as additional food source.

Following the introduction of the first adult in the insectary, we checked for mating events daily from 9:00 until 17:00. Males that were copulating, ovipositing or seen in tandem (paired individuals not copulating or ovipositing) were recorded as participating in mating events (as in Thompson et al. 2011). We identified males either directly by eye, or by using close-focusing binoculars (Kite Optics, Belgium). In the study species, the average duration of an oviposition event is ca. 90 min, and pairs remain in tandem for ca. 2 h (Banks and Thompson 1985b).

Therefore, and because pairs gathered at the small artificial pond for reproduction, we are confident that we observed all matings.

### Weather variables

The reproductive activity and survival of the study species can be influenced by weather conditions (Banks and Thompson 1987; Thompson 1990; Thompson et al. 2011), hence we obtained data of total daily precipitation (mm) and total daily sunshine (h) from the Royal Meteorological Institute of Belgium, measured by three nearby weather stations (max. distance to experimental area was 6.4 km). Weather variables were averaged over the measurements from the three weather stations. Mean daily temperature (°C) was measured using a data logger (HOBO UTBI-001, Onset Computer Corporation) installed in the outdoor insectary (see Appendix in Tüzün and Stoks 2017). Per male that mated at least once (hence where we know the duration of the maturation period and the reproductive period), we calculated two means of each of the three weather variables: (i) averaged over the individual's maturation period and (ii) averaged over its lifespan. For males that never mated, we calculated only the mean over the maturation period starting from the day of release in the insectary until the average day of first reproduction of all males that mated at least once.

### Response variables

We calculated survival as the percentage of larvae per microcosm that emerged (initial density was 30). As we could not determine the sex ratio at the start of the experiment, survival was calculated assuming an initial 1:1 sex ratio; this reflects typical initial sex ratios in damselflies (Cordero-Rivera and Stoks 2008). We defined age at emergence as the time from egg hatching to adult emergence, hence representing larval development time. Mass at emergence was quantified to the nearest 0.01 mg by weighing the one day old adults with an electronic balance (AB135-S, Mettler Toledo, Columbus, OH, USA). Note that only survival until emergence was calculated at the microcosm level (larvae were not individually marked), whereas the other response variable were calculated per individual.

Once in the insectary, we calculated lifetime mating success (LMS) separately for each male as the total number of matings during its lifespan. We quantified the duration of the maturation period as the number of days between emergence and the first day a male was seen mating, and the duration of the sexual reproductive period as the number of days between the last and first mating. Lifespan was estimated as the sum of the duration of both periods. Note that age at maturation and lifespan could only be calculated for males that mated.

## Statistical analyses

### *Analyses of treatment effects on trait means*

To test for an effect of the hatching period and urbanisation level on larval and adult traits and fitness, we constructed separate (generalized) linear mixed-effect models (GLMMs) per trait. Hatching period (early versus late) and urbanisation level (rural versus urban), as well as their interactions were included as categorical fixed effects. We also included effects of the pesticide and its interactions with hatching period and urbanisation level in all models. Yet, as pesticide exposure did not affect any of the larval or adult traits (see Appendix Tables A1 and A2), we do not report its effects in the results section to keep the manuscript focused.

Except for the LMS model with a Poisson error structure and a log link function, all models had a normal error distribution and the identity link function. As the number of larvae in microcosms may affect age and mass at emergence independent of the treatments, we included larval density per microcosm as a covariate to the corresponding models. Finally, models for all response variables measured in the insectary included weather variables as covariates. For age at first mating we included the means of the weather variables averaged over the maturation period, whereas for lifespan and LMS we included the means of the weather variables averaged over the total lifespan. All models included population (nested within urbanisation level) as a random effect. To take into account that males from the same microcosm (or population) are not independent replicates, models had additionally microcosm identity (nested within population and urbanisation level) as a random effect. The model for survival until emergence initially included population as a random effect, but given the relatively low sample size for this analysis (N = 48 microcosms), we removed the random effect after confirming it was not significant (likelihood ratio test, $P = 0.200$). Significant interactions were further investigated with contrast analyses. We report detailed result of the GLMMs in the Appendix (Tables A1 and A2).

### *Path analyses of pathways connecting treatments to adult fitness*

We used structural equation modeling (SEM, Grace 2006) to test whether any potential effect of the hatching period and urbanisation level on male LMS was operating directly, or was indirectly mediated via the metamorphic traits (age and mass at emergence). Specifically, we applied the path analysis approach which is a SEM method including only observed variables. Our data included a non-normally distributed response variable (i.e. Poisson-distributed LMS), and was hierarchically structured (i.e. nested random effects). We therefore applied the piecewise SEM (or 'generalized multilevel path analysis') approach, which allows translating a path diagram into a set of linear mixed-effect models (Shipley 2009; Lefcheck 2016).

Linear mixed effect analyses revealed significant effects of the treatments on hatching period and urbanisation level on LMS in males (see Results). Based on these results, we built three candidate models to specifically evaluate the role of direct and indirect effects of the "treatments" (i.e. hatching period and urbanisation level): (1) treatments affect fitness only directly; (2) treatments affect fitness only indirectly via the metamorphic traits; and (3) treatments affect fitness both directly and indirectly (see Fig.1 in Tüzün and Stoks 2017 for the path diagrams of the candidate models). As we additionally detected an interacting effect of hatching period and urbanisation level on mass at emergence (see results of GLMMs), we evaluated the three candidate path models (see above) separately for four subsets: we tested the effect of hatching period (as predictor in the path model) separately for only rural and for only urban males, and tested the effect of urbanisation level (as predictor in the path model) separately for early and late hatched males. All models included paths going from age and mass at emergence to LMS (see Fig.1 in Tüzün and Stoks 2017). We corrected for potential density-mediated effects by including paths going from larval density per microcosm to age and mass at emergence (results not shown). The effects on LMS remained when the weather variables were removed from the GLMMs (data not shown), therefore we did not include them in the path analysis. To capture the potential trade-off between age and mass at emergence, we included correlated errors between the two variables. When building the path models, we used the same error structure and random effects as used in the GLMMs (see above). The two categorical variables (predictors), hatching period (early = 0, late = 1) and urbanisation level (rural = 0, urban

= 1), were binary coded. Hence, a negative path going from urbanisation level to LMS, for example, should be interpreted as decreased LMS for urban males.

The most parsimonious among the three candidate path models was selected using the AICc (Akaike Information Criterion, corrected for small sample size) implemented for path models (Shipley 2013). We calculated and compared ΔAICc scores and relative support for each model (AICc weights), where lower AICc scores and higher AICc weights indicate better models (Burnham and Anderson 2002). We evaluated the overall fit of path models with Shipley's test of d-separation (Shipley 2013), which tests for missing paths in the model. Using the combined significance of these unrealized paths, a Fisher's *C* statistic is calculated and compared to a $\chi^2$ distribution to reject ($P < 0.05$) or accept ($P > 0.05$) the model. In other words, a path model with a non-significant ($P > 0.05$) Fisher's *C* statistic indicates that there were no missing pathways, and that the model supported the observed data.

All analyses were performed using R version 3.3.2 (R Development Core Team 2015). We used the 'lme4' (Bates et al. 2015) and 'nlme' (Pinheiro et al. 2016) packages for GLMMs, the 'car' package (Fox and Weisberg 2011) for testing fixed effects with Wald-$\chi^2$, the 'piecewiseSEM' package (Lefcheck 2016) for conducting path analyses, and the 'MuMIn' package (Barton 2015) to calculate AICc weights. In path models, calculating the magnitude of an indirect effect going from X to Z via Y is done by multiplying the direct path coefficients of X on Y, and of Y on Z. The total effect is the sum of all indirect and direct effects going from X to Z (Grace and Bollen 2005). We report standardized coefficients for the path models. Mass at emergence, age at maturity and lifespan were log-transformed to meet model assumptions.

## Results

Full details of the statistical results of treatment effects on metamorphic and adult traits are given in the Appendix (Tables A1 and A2).

### *Effects of treatments on metamorphic traits*

Survival until emergence was on average ca. 33%, and did not differ between any of the treatment groups (all $P > 0.05$; see Appendix Table A1 for details). Males emerged when ca. 313

days old (Fig. 1a). Late males developed ca. 26 days faster than early males ($\chi^2$ = 137.72, d.f. = 1, $P$ < 0.001; Fig. 1a) and this was not affected by the urbanisation level (period × urbanisation: $\chi^2$ = 0.65, d.f. = 1, $P$ = 0.421). Late males emerged with a smaller mass than early males when from urban populations, but not when from rural populations (period × urbanisation: $\chi^2$ = 8.62, d.f. = 1, $P$ = 0.003; contrast for hatching period: rural males: $P$ = 0.928, urban males: $P$ < 0.001; Fig. 1b). From the perspective of rural-urban differentiation, the same period × urbanisation interaction revealed that rural males emerged with a higher mass than urban males when from the late period ($P$ = 0.036), but not when from the early period ($P$ = 0.181).

### *Effects of treatments on adult traits*

On average, males became sexually mature after ca. 13.3 days (Fig. 2a). Age at maturity of males was not influenced by hatching period ($\chi^2$ = 0.09, d.f. = 1, $P$ = 0.765), urbanisation level ($\chi^2$ = 1.65, d.f. = 1, $P$ = 0.199), or their interaction ($\chi^2$ = 2.45, d.f. = 1, $P$ = 0.117). On average, mated males lived ca. 21.2 days (Fig. 2b). Lifespan of males was ca. 6 days longer in rural than urban populations in the early hatched group, but not in the late hatched group (period × urbanisation: $\chi^2$ = 4.20, d.f. = 1, $P$ = 0.040; early males: $P$ = 0.0105, late males: $P$ = 0.417; Fig. 2b). This period × urbanisation interaction also revealed that early males lived longer than late males if originating from rural populations ($P$ = 0.0115), but not from urban populations ($P$ = 0.520).

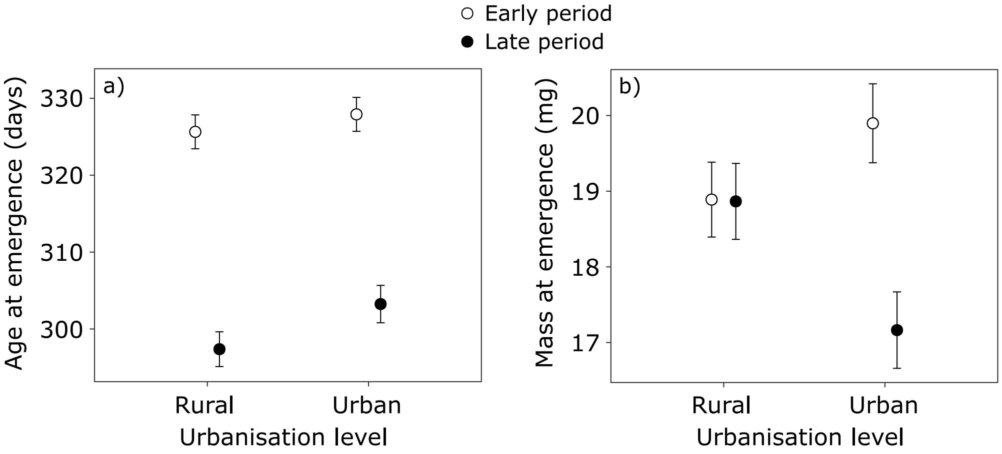

**Figure 1**. Metamorphic traits of males of the damselfly *Coenagrion puella* as a function of hatching period (open circles, early period; filled circles, late period) and urbanisation level. Age at emergence (a), mass at emergence (b). All traits are controlled for larval density in the microcosms. Given are least square means ± 1 SE.

Of the 237 adult males released in the insectary, 60 (25.3 %) mated at least once. The average number of mating was 1.143 ± 0.172 (mean ± 1 SE), and the maximum number of matings was 14 (see Appendix Table A3 for sample sizes of each treatment combination). Male lifetime mating success (LMS) was ca. two times higher in early compared to late males ($\chi^2 = 6.55$, d.f. = 1, $P = 0.010$; Fig. 2c), and ca. two times higher in rural compared to urban males ($\chi^2 = 4.89$, d.f. = 1, $P = 0.027$; Fig. 2c). Hatching period and urbanisation level did not interact in shaping LMS ($\chi^2 = 1.05$, d.f. = 1, $P = 0.305$).

*Pathways from larval treatments to adult fitness*

All path models that were selected as the most parsimonious models (based on AICc selection) were supported by the observed data (Fisher's *C* statistic: all $P > 0.08$, Appendix Tables A4 and A5). Details of the path coefficients for all path models are given in Appendix (Tables A6-A9).

For the path model with hatching period as predictor, the 'direct and indirect paths' model was the most parsimonious among the candidate models when including only rural males, whereas the 'indirect paths only' was the most parsimonious model when including only urban males. Hence, hatching period influenced male LMS both directly, and indirectly via age and mass at emergence, depending on the urbanisation level.

For rural males, there was a direct effect of hatching period on LMS, with late males having a higher chance of mating (Fig. 3a). Yet, this effect was counteracted by a negative indirect effect operating through age at emergence: late rural males had shorter larval development times, whereas longer development times resulted in an increased LMS (indirect path coefficient = -1.204). This indirect effect on LMS counteracted the positive direct effect (1.236). For urban males, there was no direct effect of hatching period on LMS (Fig. 3b). Instead, hatching period had negative indirect effects on LMS operating through age and mass at emergence: late urban males had shorter larval development times and emerged with a smaller mass, whereas longer development times and larger mass resulted in an increased LMS (total indirect effect = -0.490). The finding of a significant path between hatching period and mass at emergence in urban but not rural males is in line with the significant period × urbanisation interaction detected in the

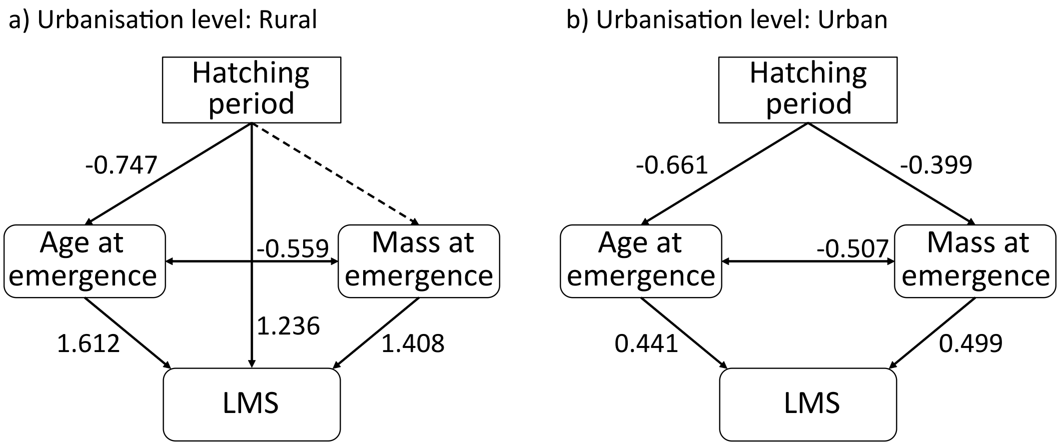

**Figure 3.** Path diagrams depicting the effects the hatching period on metamorphic traits and lifetime mating success (LMS) for the males of the damselfly *Coenagrion puella*. The most parsimonious models including only rural (a) and only urban males (b) are presented (see text for details on the model selection). Standardized path coefficients are given next to the arrows (see Tables A7 and A8 for details). The dashed line represents a non-significant path; all other paths are significant (P < 0.05). Double-headed arrows indicate correlations between traits. Binary coding for hatching period: early = 0, late = 1.

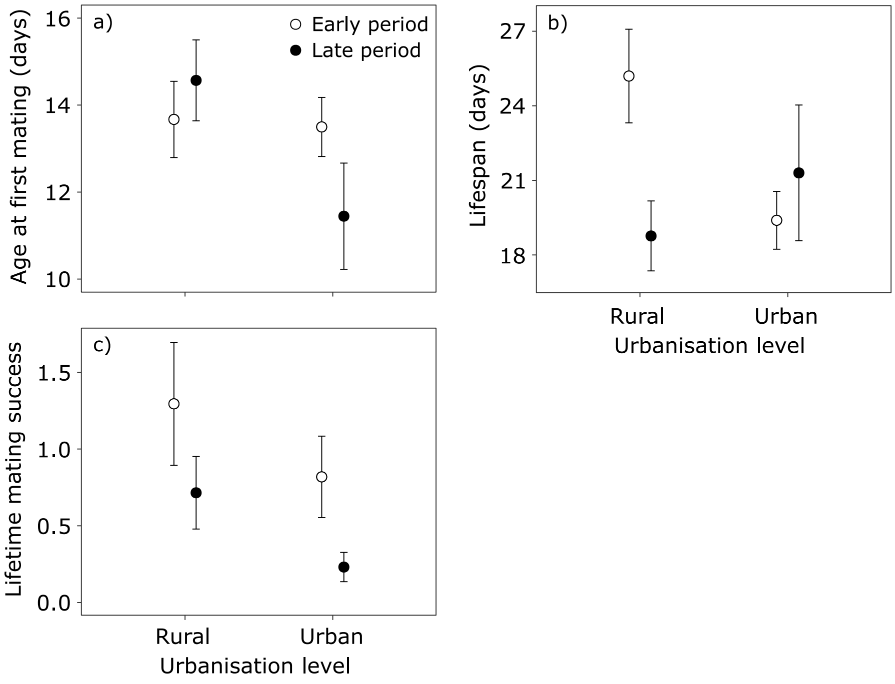

**Figure 2.** Adult traits of males of the damselfly *Coenagrion puella* as a function of hatching period (open circles, early period; filled circles, late period) and urbanisation level. Age at first mating (a), lifespan (b), lifetime mating success (c). All traits are controlled for weather conditions. Given are least square means ± 1 SE.

LMM for mass at emergence (see also Fig.1b). For both rural and urban males, there was a negative covariation between age and mass at emergence.

For the path model with urbanisation level as predictor, the ‘direct paths only’ model was the most parsimonious among the candidate models for early hatched males. For late hatched males, the ‘direct paths only’ and ‘direct and indirect paths’ models had similar support (based on AICc selection, Appendix Table A5). Yet, as none of the indirect effects in the ‘direct and indirect paths’ were significant (Appendix Table A8), we present the ‘direct paths only’ model for the pooled data instead of separate path models for early and late males. The direct effect of urbanisation level on LMS (-0.460) translated to a lower LMS in urban than in rural males (Fig. 4). As in the path models with hatching period as predictor, longer development times and higher mass at emergence resulted in an increased LMS, and there was a negative covariation between age and mass at emergence.

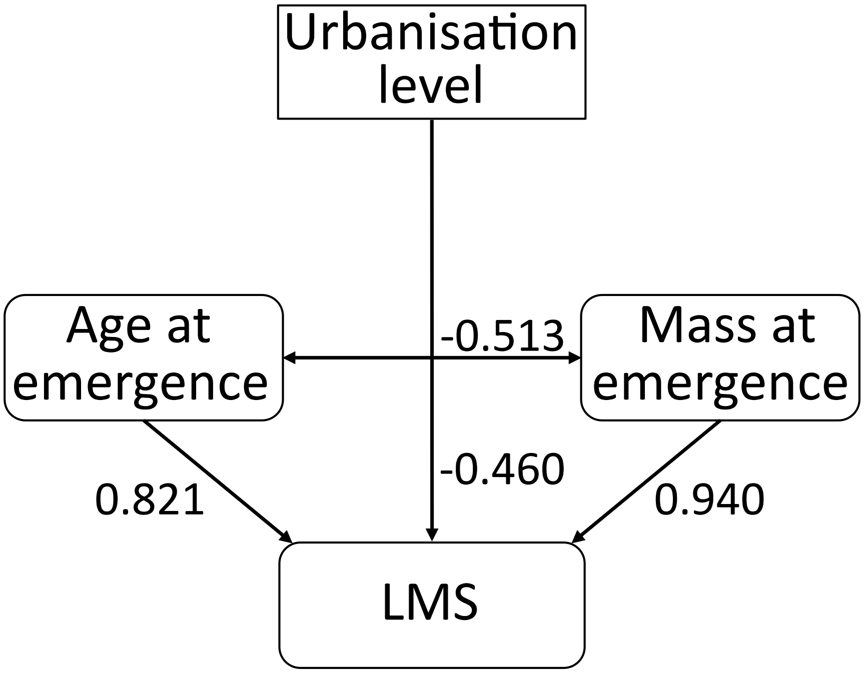

**Figure 4.** Path diagram depicting the effects the urbanisation level on metamorphic traits and lifetime mating success (LMS) for the males of the damselfly *Coenagrion puella*. The most parsimonious model is presented (see text for details on the model selection). Standardized path coefficients are given next to the arrows (see Table A9 for details). All paths are significant ($P < 0.05$). Double-headed arrows indicate correlations between traits. Binary coding for urbanisation level: rural = 0, urban = 1

## Discussion

We found strong effects of the hatching period and, to a lesser extent, of the urbanisation level on metamorphic traits, and both treatments strongly affected the lifetime mating success of *Coenagrion puella* males. The effects of hatching period and urbanisation level on life history traits strongly interacted, possibly due to the time constraints implemented by both factors. Although both treatments influenced lifetime mating success, only the hatching period-effect was mediated by the two metamorphic trait, and this depended on the urbanisation level. While the egg-to-adult survival may seem low (33%), this is high compared to estimates obtained for natural populations (e.g. 0.7% for the study species, Thompson et al. 2011).

### *Effects of the hatching period*

As predicted by life history theory on time constraints (Rowe and Ludwig 1991; Abrams et al. 1996), late-hatched males developed faster than early-hatched ones. There is ample empirical support for this pattern from a wide range of taxa (Gotthard 2001), particularly from studies with anurans (e.g. Laugen et al. 2003; Touchon et al. 2013; Orizaola et al. 2016) and odonates (e.g. De Block and Stoks 2004, 2005; Lowe et al. 2009; Mikolajewski et al. 2015). The life history acceleration in the late males likely reflects plastic responses to photoperiod. The late-hatched larvae experienced in the outdoor microcosms the same photoperiod regimes as the early-hatched larvae, yet while in an earlier larval stage; information to which larvae of the study species react by speeding up development (Mikolajewski et al. 2015). Similarly, late-hatched individuals of the study species have been shown to develop faster than early-hatched individuals in a natural population (Lowe et al. 2009). Besides plasticity, also a genetic component associated with early and late mating couples may have played a role. The faster development in late males was apparently not counterbalanced with an increased growth rate as it resulted in a smaller mass at emergence (see also Orizaola et al. 2013; Śniegula et al. 2016; for the study species: Mikolajewski et al. 2015).

Once males successfully emerged, carry-over effects of the time constraints associated with the late hatching period reduced their lifespan (rural males) and halved their LMS. The reduced lifespan may reflect a cost of fast development as recently identified in the nematode

*Caenorhabditis remanei* (Lind et al. 2017) and in the damselfly *Lestes viridis* (Janssens and Stoks, in revision). The path analysis revealed the lower LMS of late males to be present despite a direct positive effect of late hatching on LMS (in urban males). This was because of strong negative effects of late hatching operating via the lower age (rural and urban males) and mass at emergence (urban males). This fits the general pattern that smaller males have a lower mating success in damselflies (Sokolovska et al. 2000), although also the largest males may be at a disadvantage (for the study species: Banks and Thompson 1985b). Similarly, in a study with two *Rana* water frog species where time constraints were manipulated, late-hatched tadpoles metamorphosed later and at a smaller size, and had a lower post-metamorphic fitness (Altwegg and Reyer 2003). Studies with the three-spined stickleback showed that acceleration of life history due to time constraints after a growth delay caused a reduction in reproductive investment (Lee et al. 2012b, 2014, 2016), and this pattern was suggested to be a result of decreased energy allocation into reproduction (Lee et al. 2012b, 2014). The here reported negative effect of rapid development on LMS may have been operating through negative effects of rapid development on flight muscle mass. In line with this, the more time constrained high-latitude populations of the damselfly *Lestes sponsa* developed faster than southern populations, yet suffered a lower flight muscle mass (Śniegula et al. 2017). The flight muscle mass is a key component of insect flight performance (Marden 1989; Berwaerts et al. 2002), which enhances mating probability in the study species (Gyulavári et al. 2014; Tüzün et al. 2017b).

### *Effects of the urbanisation level*

Rural populations are expected to be under stronger time constraints than urban populations because of the shorter thermally favourable larval growth season (due to the “urban heat island effect” in urban ponds, Somers et al. 2013; Yang et al. 2013; Zipper et al. 2016; for ponds in the study region: Brans et al. 2018). In line with this, using a common garden experiment in the laboratory, we recently documented consistently faster growth rates of rural than urban larvae of *C. puella* along a temperature gradient (Tüzün et al. 2017a). A faster growth in rural larvae likely caused their higher mass at emergence compared to urban individuals when under the additional time constraints imposed by late hatching.

A striking finding was that rural males had a twice higher LMS than urban males. No other studies compared LMS between urban and rural populations, but related effects were found for reproduction. Studies with passerine birds reported a decreased productivity in urban populations compared to rural populations (Chamberlain et al. 2009). The lower reproductive success in urban populations of great tits could be explained by a reduced clutch size (operating via earlier breeding times in cities) and a reduced nestling body mass (Sprau et al. 2017). On the other hand, urban populations from an orb-weaving spider had a higher fecundity compared to populations from rural areas, which was explained by the higher temperatures and prey availability in urban habitats (Lowe et al. 2014). Our finding that the urbanisation effect on LMS was not operating via age or mass at emergence indicates alternative routes for this pathway. Following the significant main effect of urbanisation level on lifespan, we added lifespan as a covariate to the GLMM explaining variation in LMS (excluding non-mated males, for which we have no accurate lifespan data), where it had a significant effect on LMS, while the effect of urbanisation level was no longer significant (Table A10). This suggests the longer lifespan of mated rural males might be driving the differentiation in LMS. In general, lifespan is a major determinant of LMS in male damselflies (e.g. Fincke 1982; Stoks 2000), including the study species (Thompson et al. 2011).

Given that rapid growth is expected to negatively covary with lifespan (Metcalfe and Monaghan 2003; Lee et al. 2012a), our finding of a longer lifespan of the faster growing rural individuals (Tüzün et al. 2017a) seems counterintuitive at first. Yet, a recent study using the closely related damselfly *Ischnura elegans* similarly reported longer lifespan of the faster growing French populations compared to the slower growing Swedish populations (see Appendix S3 in Debecker et al. 2017), and metabolic uncoupling (Speakman et al. 2004) was suggested as an explanation for this pattern. Alternatively, a higher extrinsic mortality might be the driver of the shorter lifespan, hence lower LMS, in urban males as life expectancy decreases with an increase in the risk of extrinsic mortality (Monaghan et al. 2008). Urban habitats may impose a higher extrinsic mortality in adult damselflies, e.g. due to more frequent and intense heat waves (Ward et al. 2016; for the study region: Wouters et al. 2017) that may cause death because of desiccation (for odonates: Corbet 1999), and more intense traffic causing road kills (for odonates:

Muñoz et al. 2015). On a related note, a recent study in great tits showed genetically (or epigenetically) inherited shorter telomere length (widely associated with reduced lifespan and fitness, Monaghan and Haussmann 2006) in urban populations (Salmón et al. 2016), which might possibly explain our finding of longer lifespan and higher LMS in rural males. Although studies on the telomere length–lifespan association in insects are extremely rare, Jemielity et al. (2007) report that short-lived males of the ant *Lasius niger* had shorter telomeres (but see Walter et al. 2007 for no correlation in *Drosophila melanogaster*).

### *Conclusions*

Our powerful longitudinal experiment that integrated an outdoor microcosm part and a field insectary part allowed us to identify effects of hatching period and urbanisation level from the egg stage across metamorphosis up to lifetime mating success under seminatural conditions. Key findings of this approach were that both the hatching period and the urbanisation level shaped adult fitness, yet through different pathways. Only the effect of hatching period was mediated by changes in age and mass at emergence, matching the general assumption of life history theory predicting how conditions experienced in the larval stage should affect adult fitness (Rowe and Ludwig 1991; Abrams et al. 1996). Noteworthy, there was also a positive direct effect of late hatching on lifetime mating success in rural males which was, however, cancelled out by the negative indirect effects via the metamorphic traits. This clearly illustrates the importance of disentangling direct and indirect effects on fitness, which was possible with the path analysis approach. The importance of carry-over effects in ecology is becoming increasingly clear (Harrison et al. 2011; O’Connor et al. 2014). Our results add to this by demonstrating that the widespread natural variation in hatching period in animal populations (e.g. Altwegg 2002; De Block and Stoks 2005; Orizaola et al. 2013) may have long-term carry-over effects and reduce adult lifespan and lifetime mating success the next summer.

Our study is the first to apply a common garden rearing experiment to test for urbanisation-driven differentiation in lifetime mating success (but see Kaiser et al. 2016 for a split-brood experiment comparing survival and mass at emergence in rural, semiurban and urban populations of two butterfly species). Although we cannot fully exclude long-term maternal effects acting across metamorphosis, it is likely that the here reported differentiation in LMS between rural and urban damselflies has (at least partly) a genetic basis. Our findings of a twice lower LMS in urban populations are important for the study of population differentiation and adaptation to urbanisation (Alberti et al. 2017a), by adding to the limited evidence showing fitness costs of adjusting to an urban life (e.g. Salmón et al. 2016; Sprau et al. 2017; Brans et al. 2017a,b).

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## Appendix

**Table A1.** Results of the linear model testing for the effects of hatching period, urbanisation level and pesticide treatment on survival until emergence of the damselfly *Coenagrion puella.*

| | Survival | | |
|---|---|---|---|
| | df | F | *P* |
| Hatching period (Period) | 1 | 0.37 | 0.543 |
| Urbanisation level (Urban) | 1 | 0.90 | 0.349 |
| Pesticide treatment (Pesticide) | 1 | 0.37 | 0.543 |
| Period × Urban | 1 | 0.90 | 0.349 |
| Period × Pesticide | 1 | 3.80 | 0.058 |
| Urban × Pesticide | 1 | 0.03 | 0.868 |
| Period × Urban × Pesticide | 1 | 1.12 | 0.296 |
| Residuals | 40 | | |

**Table A2.** Results of the (generalized) linear mixed effect models testing for the effects of hatching period, urbanisation level and pesticide treatment on metamorphic traits (age at emergence, mass at emergence) and adult traits (age at maturation, lifespan, lifetime mating success) for the males of the damselfly *Coenagrion puella.*

| | Age at emergence | | Mass at emergence | | Age at maturation | | Lifespan | | Lifetime mating success | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* | $\chi^2$ | *P* |
| Hatching period (Period) | **137.72** | **<0.001** | **8.30** | **0.004** | 0.09 | 0.765 | 2.39 | 0.122 | **6.55** | **0.010** |
| Urbanisation level (Urban) | 3.17 | 0.075 | 0.44 | 0.508 | 1.65 | 0.199 | **4.16** | **0.041** | **4.89** | **0.027** |
| Pesticide treatment (Pesticide) | 3.63 | 0.057 | 1.26 | 0.262 | 0.43 | 0.513 | 0.13 | 0.713 | 0.27 | 0.605 |
| Period × Urban | 0.65 | 0.421 | **8.62** | **0.003** | 2.45 | 0.117 | **4.20** | **0.040** | 1.05 | 0.305 |
| Period × Pesticide | 0.26 | 0.610 | 0.72 | 0.397 | 0.11 | 0.734 | 0.18 | 0.672 | 0.18 | 0.669 |
| Urban × Pesticide | 0.03 | 0.871 | 1.62 | 0.203 | 0.28 | 0.598 | 0.72 | 0.399 | 0.01 | 0.921 |
| Period × Urban × Pesticide | 0.22 | 0.636 | 2.43 | 0.119 | 0.00 | 0.995 | 1.15 | 0.283 | 0.06 | 0.808 |
| Larval density in microcosm | **11.10** | **0.001** | **40.79** | **<0.001** | | | | | | |
| Temperature | | | | | **66.87** | **<0.001** | **21.92** | **<0.001** | **12.77** | **<0.001** |
| Precipitation | | | | | 0.06 | 0.804 | 0.43 | 0.513 | 0.09 | 0.758 |
| Sunshine | | | | | **4.82** | **0.028** | **5.90** | **0.015** | **4.48** | **0.034** |

Significant effects ($P < 0.05$) are indicated in bold. See text for a detailed description of the models.

**Table A3.** Number of male damselflies released into the insectary, presented per treatment combination. Numbers in brackets represent number of adults that mated at least once.

| | | Hatching period | |
|---|---|---|---|
| | | Early | Late |
| Urbanisation level | Rural | 62 (15) | 65 (22) |
| | Urban | 62 (18) | 48 (5) |

**Table A4.** Parameters for model fits and model selection with hatching period as predictor. Candidate path models were tested separately for only rural and only urban males. Models where the Fisher's *C* statistic are not significant (*P* > 0.05) have a good fit.

| Candidate path models | Fisher's *C* | d.f. | *P* | K | AICc | ΔAICc | ωAICc |
|---|---|---|---|---|---|---|---|
| *Urbanisation level: Rural* | | | | | | | |
| Direct paths only | 37.22 | 6 | <0.001 | 16 | 74.165 | 27.032 | <0.001 |
| Indirect paths only | 33.18 | 4 | <0.001 | 17 | 72.795 | 25.662 | <0.001 |
| Direct + indirect paths | **4.80** | **2** | **0.091** | **18** | **47.133** | **0** | **0.999** |
| *Urbanisation level: Urban* | | | | | | | |
| Direct paths only | 44.25 | 6 | <0.001 | 16 | 82.099 | 40.917 | <0.001 |
| Indirect paths only | **0.53** | **4** | **0.970** | **17** | **41.182** | **0** | **0.805** |
| Direct + indirect paths | 0.50 | 2 | 0.780 | 18 | 44.016 | 2.834 | 0.195 |

Best supported models are indicated with bold. K=likelihood degrees of freedom, AICc=Akaike's Information Criterion corrected for sample size, ΔAICc=difference in AICc between best model and other candidate models, ωAICc= Akaike weights, representing relative support for model.

**Table A5.** Parameters for model fits and model selection with urbanisation level as predictor. Candidate path models were tested separately for only early and only late males. For the pooled data, the 'direct paths only' is presented (see main text for details).

| Candidate path models | Fisher's *C* | d.f. | *P* | K | AICc | ΔAICc | ωAICc |
|---|---|---|---|---|---|---|---|
| *Hatching period: Early* | | | | | | | |
| Direct paths only | **3.36** | **6** | **0.762** | **16** | **40.444** | **0** | **0.715** |
| Indirect paths only | 5.25 | 5 | 0.262 | 17 | 45.024 | 4.580 | 0.072 |
| Direct + indirect paths | 0.36 | 2 | 0.835 | 18 | 42.874 | 2.430 | 0.212 |
| *Hatching period: Late* | | | | | | | |
| Direct paths only | **11.22** | **6** | **0.082** | **16** | **48.887** | **1.200** | **0.325** |
| Indirect paths only | 11.18 | 4 | 0.025 | 17 | 51.622 | 3.935 | 0.083 |
| Direct + indirect paths | **4.41** | **2** | **0.110** | **18** | **47.687** | **0** | **0.592** |
| *Pooled* | | | | | | | |
| Direct paths only | 2.26 | 4 | 0.689 | 16 | 36.733 | - | - |

Best supported models are indicated with bold. K=likelihood degrees of freedom, AICc=Akaike's Information Criterion corrected for sample size, ΔAICc=difference in AICc between best model and other candidate models, ωAICc= Akaike weights, representing relative support for model.

**Table A6.** Standardized path coefficients for the best supported path model ('direct and indirect paths') explaining effects of the hatching period on lifetime mating success of male damselflies, including only rural males.

| Response | Predictor | Coefficient | SE | *P* |
|---|---|---|---|---|
| Lifetime mating success | Hatching period | 1.236 | 0.239 | <0.001 |
| Lifetime mating success | Age at emergence | 1.612 | 0.199 | <0.001 |
| Lifetime mating success | Mass at emergence | 1.408 | 0.146 | <0.001 |
| Age at emergence | Hatching period | -0.747 | 0.087 | <0.001 |
| Age at emergence | Larval density in microcosm | 0.087 | 0.078 | 0.279 |
| Mass at emergence | Hatching period | -0.001 | 0.116 | 0.992 |
| Mass at emergence | Larval density in microcosm | -0.408 | 0.106 | 0.001 |
| Age at emergence | Mass at emergence | -0.559 | | <0.001 |

The last row represents the partial correlation between age and mass at emergence.

**Table A7.** Standardized path coefficients for the best supported path model ('only indirect paths') explaining effects of the hatching period on lifetime mating success of male damselflies, including only urban males.

| Response | Predictor | Coefficient | SE | *P* |
|---|---|---|---|---|
| Lifetime mating success | Age at emergence | 0.441 | 0.164 | 0.007 |
| Lifetime mating success | Mass at emergence | 0.499 | 0.164 | 0.002 |
| Age at emergence | Hatching period | -0.661 | 0.082 | <0.001 |
| Age at emergence | Larval density in microcosm | 0.268 | 0.078 | 0.003 |
| Mass at emergence | Hatching period | -0.390 | 0.103 | 0.001 |
| Mass at emergence | Larval density in microcosm | -0.493 | 0.094 | <0.001 |
| Age at emergence | Mass at emergence | -0.507 |  | <0.001 |

The last row represents the partial correlation between age and mass at emergence.

**Table A8.** Standardized path coefficients for the best supported path model ('direct and indirect paths') explaining effects of the urbanisation level on lifetime mating success of male damselflies, including only late hatched males.

| Response | Predictor | Coefficient | SE | *P* |
|---|---|---|---|---|
| Lifetime mating success | Urbanisation level | -0.521 | 0.279 | 0.062 |
| Lifetime mating success | Age at emergence | 0.838 | 0.158 | <0.001 |
| Lifetime mating success | Mass at emergence | 1.388 | 0.203 | <0.001 |
| Age at emergence | Urbanisation level | 0.150 | 0.106 | 0.251 |
| Age at emergence | Larval density in microcosm | 0.043 | 0.104 | 0.683 |
| Mass at emergence | Urbanisation level | -0.255 | 0.124 | 0.132 |
| Mass at emergence | Larval density in microcosm | -0.380 | 0.113 | 0.004 |
| Age at emergence | Mass at emergence | -0.651 |  | <0.001 |

The last row represents the partial correlation between age and mass at emergence.

**Table A9.** Standardized path coefficients for the best supported path model ('only direct paths') explaining effects of the urbanisation level on lifetime mating success of male damselflies. Note that the model uses pooled data instead of separate path models for early and late males (see main text for details).

| Response | Predictor | Coefficient | SE | *P* |
|---|---|---|---|---|
| Lifetime mating success | Urbanisation level | -0.460 | 0.001 | <0.001 |
| Lifetime mating success | Age at emergence | 0.821 | 0.001 | <0.001 |
| Lifetime mating success | Mass at emergence | 0.940 | 0.001 | <0.001 |
| Age at emergence | Larval density in microcosm | 0.098 | 0.107 | 0.366 |
| Mass at emergence | Larval density in microcosm | -0.459 | 0.078 | <0.001 |
| Age at emergence | Mass at emergence | -0.513 |  | <0.001 |

The last row represents the partial correlation between age and mass at emergence.

**Table A10.** Results of the linear mixed effect model with lifespan as covariate, testing for the effects of hatching period, urbanisation level and pesticide treatment on lifetime mating success for the males of the damselfly *Coenagrion puella.* Note that this analysis excludes non-mated males, as lifespan was calculated only for mated males (see main text for details).

|  | Lifetime mating success |  |
|---|---|---|
|  | $\chi^2$ | *P* |
| Hatching period (Period) | 0.05 | 0.825 |
| Urbanisation level (Urban) | 2.62 | 0.106 |
| Pesticide treatment (Pesticide) | 1.68 | 0.195 |
| Period × Urban | 0.17 | 0.682 |
| Period × Pesticide | 0.56 | 0.455 |
| Urban × Pesticide | 1.82 | 0.177 |
| Period × Urban × Pesticide | 0.95 | 0.329 |
| **Lifespan*** | **75.84** | **<0.001** |
| **Temperature** | **60.65** | **<0.001** |
| Precipitation | 0.03 | 0.848 |
| **Sunshine** | **14.07** | **<0.001** |

Significant effects (*P* < 0.05) are indicated in bold. * The slope for lifespan was 0.974 (SE = 0.112).

# CHAPTER 6

# Sexual selection reinforces a higher flight endurance in urban damselflies

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## Abstract

Urbanisation is among the most important and globally rapidly increasing anthropogenic processes, and is known to drive rapid evolution. Habitats in urbanised areas typically consist of small, fragmented and isolated patches, which is expected to select for a better locomotor performance, along with its underlying morphological traits. This, in turn, is expected to cause differentiation in selection regimes, as populations with different frequency distributions for a given trait will span different parts of the species' fitness function. Yet, very few studies considered differentiation in phenotypic traits associated with patterns in habitat fragmentation and isolation along urbanisation gradients, and none considered differentiation in sexual selection regimes. We investigated differentiation in flight performance and flight-related traits, and sexual selection on these traits across replicated urban and rural populations of the scrambling damselfly *Coenagrion puella*. To disentangle direct and indirect paths going from phenotypic traits over performance to mating success, we applied a path analysis approach. We report for the first time direct evidence for the expected better locomotor performance in urban compared to rural populations. This matches a scenario of spatial sorting, whereby only the individuals with the best locomotor abilities colonise the isolated urban populations. The covariation patterns and causal relationships among the phenotypic traits, performance and mating success strongly depended on the urbanisation level. Notably, we detected sexual selection for a higher flight endurance only in urban populations, indicating that the higher flight performance of urban males was reinforced by sexual selection. Taken together, our results provide a unique proof of the interplay between sexual selection and adaptation to human-altered environments.

## Introduction

Urbanisation is a major human-induced selective force driving rapid phenotypic change and evolution (Alberti 2015; Alberti et al. 2017a; Sullivan et al. 2017). Urbanised areas typically consist of small, fragmented and isolated patches (Parris 2016). In addition, urban roads with heavy traffic can act as strong barriers to the movement of many organisms, including flying insects (Muñoz et al. 2015). As a result, urban habitats will likely not be colonised by a random set of immigrants from rural populations (e.g. Evans et al. 2009). Surprisingly few studies, however, considered differentiation in phenotypic traits associated with patterns in habitat fragmentation and isolation along urbanisation gradients (but see Cheptou et al. 2008; Gomez and Van Dyck 2012; Schoville et al. 2013; Dubois and Cheptou 2017).

One trait frequently studied in relation to habitat fragmentation and isolation in general is dispersal ability (Baguette et al. 2012; Cote et al. 2017). Traits related to dispersal ability such as locomotor performance (e.g. Phillips et al. 2006) and its underlying morphological traits are usually increased in fragmented and isolated populations (Van Dyck and Matthysen 1999; Baguette et al. 2012). However, depending on the spatial resource distribution, mobility can also be reduced in fragmented landscapes (Bergerot et al. 2012). Colonisation of empty patches is a process tightly associated with dispersal ability. Consequently, similar findings have been reported in studies comparing edge and core populations of range expanding species: increased dispersal ability is common in the more recently colonised edge populations (Hill et al. 2011). Both spatial sorting (Shine et al. 2011), the process where only the organisms with the best locomotor abilities end up at the range front, and local adaptation (Travis and Dytham 2002) may contribute to dispersal-enhancing phenotypes at invasion fronts, although spatial sorting has been suggested as the main driver (Van Petegem et al. 2016). Considering that urban habitats are colonised by rural source populations (e.g. Evans et al. 2009), and given the typically fragmented and isolated urban habitats (e.g. Luck and Wo 2002; Cane et al. 2006), only the best dispersers from the rural populations are likely to enter urban habitats. Therefore, we could expect an increased locomotor performance and associated phenotypic traits in urban populations.

Changes in phenotypes caused by evolution may generate feedback loops from evolution to ecology (Hendry 2016). One such important, but relatively understudied by-product of differences in mean phenotypic traits between populations is its effect on the direction and strength of selection. Populations with different frequency distributions for a given trait will span different parts of the species' fitness function. When the fitness function of the species is non-linear, this will result in different selection patterns in the different populations (Endler 1986; Figure 6.5 in Conner and Hartl 2004). Therefore, the human-induced changes in mean phenotypic traits in urban habitats (Alberti et al. 2017a; Sullivan et al. 2017) can be expected to result in different selection regimes between urban and rural populations.

This may be especially true for sexual selection in mating systems such as scrambling competition where increased locomotor performance is selected for (Husak and Fox 2008). We therefore hypothesize the higher mean values of locomotor traits that are to be expected in urban populations to generate different sexual selection patterns on these traits.

We investigated differentiation in flight performance and flight-related traits, and sexual selection on these traits between urban and rural populations of the scrambling damselfly *Coenagrion puella*. Due to their conspicuous mating behaviour localized at pond margins, damselflies are ideal study organisms to study sexual selection in natural populations (Córdoba-Aguilar 2008). We quantified flight performance (flight endurance and flight speed) and measured a set of flight-related phenotypic traits including wing morphology and physiology (relative fat content and flight muscle ratio) of mated and unmated males. To reveal the covariation patterns between the flight-related traits, flight performance and mating success, and whether these differed between urban and rural populations, we used a path analysis approach; a powerful tool to investigate sexual selection (Kingsolver and Schemske 1991). We expected sexual selection for a higher flight endurance, as this increases the chances of encountering mates in scrambling species (Husak and Fox 2008). In line with this, we expected increased investment in morphological and physiological traits enhancing flight performance in mated males, although costs associated with mating may obscure patterns of sexual selection on these traits (e.g. energy reserves: Blanckenhorn et al. 2003, 2004). Based on the characteristics of urban habitats discussed above (Parris 2016), we predicted urban males to show a higher flight performance and associated phenotypic traits compared to rural males. Given that sexual selection patterns can be shaped by mean phenotypic trait values (Endler 1986), we predict differentiation in sexual selection on flight performance between urban and rural populations. Given the expected higher flight endurance in urban populations, and the finding that flight endurance only positively influences mating success above a threshold value in the study species (Gyulavári et al. 2014), we predicted stronger sexual selection on flight endurance in urban populations.

## Materials and methods

### *Study populations and sampling*

*Coenagrion puella* is one of the most common damselflies in Europe (Dijkstra and Lewington 2006), occupying ponds in both rural and urban areas (Goertzen and Suhling 2013). We studied three rural populations (Bierbeek, Bornem and Houwaart) and three urban populations (Leuven, Mechelen and Oudenaarde). All six populations were situated within a 45 km radius in Flanders, Belgium. We used a two-step procedure using Geographic Information System (GIS) for the selection of urban and rural ponds. First, we selected three urban plots with >15% built-up area, and three rural plots with <3% built-up area, all 3 x 3 km. Next, we selected a subplot of 200 x 200 m in each plot, with the same urbanisation level following the same criteria of percentage built-up area. This way we made sure that both the direct environment, represented by subplots, and the broader surroundings, represented by plots, reflected the same urbanisation level. This sampling design was also applied in a recent study by Piano et al. (2017).

Males of *C. puella* search for females by patrolling low at the breeding pond and display scramble competition for mates. Female choice behaviour is thought to be not important in this species (Banks and Thompson 1985). To assess sexual selection on traits, we sampled mated and unmated males in all six populations during July 2013. Comparing trait values between sets of mated and unmated males is an often used method to detect sexual selection (e.g. Blanckenhorn et al. 1999; Gosden and Svensson 2008). We categorized males as mated when they were caught in tandem position or copulation. Unmated males were those not associated with a female but that were active at the reproduction site. As the average duration of association between a couple is ca. 2 h (Banks and Thompson 1985), and we collected all individuals between 11:00 and 15:00 h when sexual activity peaks, we lowered the probability of wrongly categorizing a male as unmated that would have mated that day. Yet, we cannot fully exclude that we might have missed matings on a given day and that our sample of ‘unmated’ males contained males that would have mated that day. Nevertheless, this would make our results of sexual selection conservative as it would introduce noise in the dataset and make it harder to detect phenotypic differences between our samples of mated and unmated males. Despite this limitation, this technique has been successfully applied to detect signals of sexual selection in damselflies (e.g. Gosden and Svensson 2008), including the study species (Gyulavári et al. 2014). In total 576 males (292 mated, 284 unmated) were collected on sunny days near the border of their breeding pond (Table S1). All males were transferred individually to the laboratory in 50 ml plastic cups (diameter: 3.5 cm; height: 7 cm), and were maintained in an incubator at 14 °C until the start of the flight test. To control for potential effects of the time span between capture and the flight test, we recorded the time of capture of each individual.

*Flight test*

We tested the flight performance (flight speed and flight endurance) of individuals following the methodology used before in damselflies (Gyulavári et al. 2014; Therry et al. 2014b). Flight performance traits were quantified when animals were flying in a plexiglass flight tube (diameter: 50 cm; height: 200 cm) in a temperature controlled room (21.5 ± 0.5 °C). The individual to be tested was placed in its cup at the bottom of the flight tube, and was allowed to acclimatize for 3 min. To initiate the flight test, the cup was gently rotated horizontally until the individual took off. All flight tests were conducted on the day of capture.

A typical flight bout consisted of an initial, fast and linear upward trajectory, followed by a slower trajectory where the individual stepwise ascended and eventually reached the highest vertical distance of its flight trajectory. The frictionless surface of the tube prevented individuals from resting on the tube walls. Hence, a flight bout ended when the individual landed on the bottom of the flight tube. We recorded the following parameters during each flight bout: (1) the height reached during the initial trajectory ('initial height'), (2) the duration of this initial trajectory ('initial duration'), and (3) the total duration of the flight bout ('total duration'). The flight tube was graded every 10 cm to allow estimating the height parameters. The flight durations were measured using a chronometer (accuracy 0.01 s). We estimated flight speed as 'initial height'/'initial duration', whereas 'total duration' was interpreted as flight endurance. Each individual was tested only once. Flight performance tested this way has been shown to be repeatable in another damselfly (Gyulavári et al. 2017) and to be related to mating status in the field (Gyulavári et al. 2014; Therry et al. 2014b).

To correct for potential influences of body temperature on flight performance, we measured the thorax temperature of individuals to the nearest 0.1 °C with a micro-thermocouple (BAT-12 type, Physitemp Instruments, Clifton NJ) directly after the flight test. We also counted the number of mites attached to individuals that were tested for their flight performance, as water mites have been shown to negatively influence flight ability (e.g. Nagel et al. 2010) and mating success in male damselflies (Forbes and Robb 2008), including the study species (Thompson et al. 2011). Due to time constraints, we performed the flight test on a subset of individuals collected on a given day (total N = 338, see Table S1). Individuals with damaged wings were not tested.

### *Flight-related physiological traits*

We measured two flight-related physiological traits of all collected males, based on the protocol by Swillen et al. (2009): relative fat content and relative flight muscle mass. After removing wings and legs, we separated the head, thorax and abdomen using scissors. The thorax of each individual was placed separately in Eppendorf tubes, dried for 48 h at 60 °C, and weighted to the nearest 0.01 mg using a microbalance. To extract the fat, we added 1.5 ml dichloromethane (99%) to the Eppendorf tubes, and placed them on an automatic shaker for 24 h. After removal of the dichloromethane with the dissolved fat, we dried the body parts for another 48 h at 60 °C and weighed them again. Fat content was calculated by subtracting the dry masses before and after fat extraction. To obtain muscle mass, we added 1.5 ml NaOH (0.35 M) to the Eppendorf tubes to break down all muscle tissue. Finally, we placed the tubes on a shaker for 24 h, and after removal of the NaOH with dissolved muscle tissue we weighted the dried thorax without muscles. Flight muscle mass was quantified as the difference in dry mass of the thorax before and after this procedure; the flight muscles of odonates make up most of the thorax (Marden 1989). Relative fat content was calculated as the ratio of fat mass in the thorax to body dry mass, whereas flight muscle ratio was calculated as the ratio of muscle mass to thorax dry mass.

### *Wing morphometrics*

We quantified flight-related wing characteristics using geometric morphometrics (Rohlf and Slice 1990). We photographed the left hind wing of all males, and digitized the images in the tpsDig2 software (Rohlf 2015). To capture a detailed wing shape, we placed 7 landmarks along the wing outline where it is intersected by major wing veins, and 5 semi-landmarks where intersections were not consistent between wings (Fig. S1). The landmark coordinates were imported into the software tpsRelw (Rohlf 2015), where they were subjected to a generalized procrustes analysis in order to remove any non-shape related differences due to variation in position, orientation and size (Rohlf and Slice 1990). During this process, semi-landmarks are allowed to slide along their tangent vectors in order to minimize the shape differences between specimens. After this step, the consensus conformation was compared with each specimen to generate shape variables termed partial warps. Finally, a principal component analysis was performed on these partial warp scores to compute relative warps. The first three relative warps explained 81.4% of the wing shape variation (relative warp 1 = 59.7%, relative warp 2 = 12.3%, relative warp 3 = 9.4%), and further analyses were conducted with these three relative warps. We visualized changes in wing shapes associated with increasing and decreasing relative warp scores by presenting transformation grids (Fig. S3) using the R package 'geomorph' (Adams and Otárola-Castillo 2013).

Wing size was estimated as centroid size, the square root of the summed squared distances from each landmark to the geometric centre of each wing (Bookstein 1991). As centroid size and body size are strongly correlated in damselflies (e.g. Outomuro et al. 2011), we used wing centroid size as a proxy for body size (e.g. Outomuro et al. 2013, 2016). Finally, wing loading was measured as the ratio of total wet body mass to wing area, which was calculated as the polygon area enclosed by the landmarks.

### *Statistical analyses*

To test for an effect of urbanisation level (urban versus rural) on the physiological (i.e. relative fat content and flight muscle ratio), wing morphological (i.e. centroid size, wing loading and three relative warps) and flight performance (i.e. flight endurance and speed) traits, we constructed separate linear mixed-effect models per response variable. We included mating status (unmated versus mated) and its interaction with urbanisation level as an additional term in all models to control for potential variation in the traits due to mating status. All models had a normal error structure and the identity link, and included population (nested within urbanisation level) as a random effect.

To investigate for effects of the morphological and physiological traits on mating success, whether these effects are direct or indirectly mediated via flight performance, and differed between urban and rural males, we used a path analysis approach (Grace 2006). This is a special case of structural equation modelling (SEM) that contains only observed variables. SEM allows to explore covariation patterns and causal relationships among variables (Grace 2006). More specifically, we applied the generalized multilevel path analysis approach (Shipley 2009) which allows for the implementation of non-normally distributed (here binomial values of mating success), hierarchically structured data (here nested random effect of population), which cannot be applied in the traditional variance-covariance based SEM. This method has recently been successfully applied in several studies (e.g. Laliberte et al. 2014; Jing et al. 2015; Duffy et al. 2016; Theodorou et al. 2016).

We constructed an *a priori* path model (Fig. S2) based on previous research that investigated relationships between physiological and morphological traits and flight performance and/or mating success in damselflies (Grether 1996; De Block and Stoks 2007; Gosden and Svensson 2008; Swillen et al. 2009; Steele et al. 2011; Gyulavári et al. 2014; Therry et al. 2014b; Outomuro et al. 2016; Gyulavári et al. 2017). As relative warps are unique shape variables that cannot be compared between studies, we could not predict their effect on the here used response variables. Hence, we included all possible paths between the three relative warps and the response variables (i.e. both flight performance traits and mating success) in the *a priori* path model. We also added the correlation between wing centroid size and wing loading to the path model. In addition, we added correlations between wing centroid size and the three relative warps to account for allometric effects.

To account for non-linear relationships in the path model, we conducted an exploratory step where we tested for potentially interesting quadratic effects. We ran separate mixed-effect models with either flight endurance, flight speed or mating success as response variable, and the linear term of a given trait as fixed effect. We then included the quadratic term of the trait as an additional fixed effect, and compared the AIC values of the two models (i.e. model with linear vs model with linear + quadratic fixed effect). We selected the quadratic relationship for the path model if the model with the quadratic term decreased the AIC score by 2 or more (i.e. resulted in a better supported model, Jørgensen 2004). Based on these exploratory analyses (data not shown), we included four additional paths to our *a priori* model: the quadratic term of relative fat content and centroid size with flight endurance as response variable, and the quadratic term of the second relative warp and centroid size with mating status as response variable.

We analysed the *a priori* path model by translating the path diagram into a set of linear mixed-effect models (following Shipley 2009): (1) flight endurance regressed against physiological and morphological traits; (2) flight speed regressed against physiological and morphological traits; and (3) mating success regressed against flight performance, physiological and morphological traits. In addition, we tested whether the following variables had an effect on flight performance by including them as covariates to the models: number of mites, body temperature, time of the flight test, and time span between capture and flight test. We also tested whether number of mites had an influence on mating success. None of these covariates had a significant effect (all $P > 0.13$), and they were excluded from further analyses. All models incorporated population (nested within urbanisation level) as random effect. This approach takes into account that individuals from the same populations are not independent replicates, thereby avoiding pseudoreplication. Flight performance variables were modelled with linear mixed-effect models and had a normal error structure and the identity link, whereas mating success was modelled with generalized linear mixed-effect models with a binomial error structure and a logit-link function. Flight endurance, flight speed and wing centroid size were log-transformed. All continuous variables were standardized (i.e. mean-centred and divided by the standard deviation). As we had directional hypotheses for the relationship between flight performance and mating success (i.e. increased mating success with higher flight performance, Gyulavári et al. 2014; Therry et al. 2014b; Gyulavári et al. 2017), we report one-sided *P*-values for the corresponding path coefficients. For the other paths coefficients (i.e. paths going from physiological and morphological traits to flight performance and mating success), we report two-sided *P*-values.

In order to test whether the relationships between variables differed between the two urbanisation levels, we then evaluated the *a priori* path model separately for urban and rural populations (see Minden et al. 2016; Theodorou et al. 2016 for a similar approach). In addition, we directly compared the path coefficients derived from the two separate path models (i.e. only urban vs only rural) using the standard approach for comparing regression coefficients (Zar 1999; see Dingemanse et al. 2010 for a similar approach). We compared paths coefficients that had a $P < 0.05$ in either urban or rural models. For acquiring goodness-of-fit indices of the path models, we applied Shipley's d-sep test (Shipley 2002) which tests for any unrealised paths in the model, and provides Fisher's $C$ statistics where the significance of unrealised paths is combined (see Shipley 2009 for the calculations). By comparing the Fisher's $C$ statistics to a $\chi^2$ distribution, we rejected or accepted each of the three models (i.e., models with $P > 0.05$ were accepted).

All analyses were performed using R version 3.2.3 (R Development Core Team 2015). We used the 'lme4' package (Bates et al. 2015) for mixed-effect models, and the 'piecewiseSEM' package (Lefcheck 2016) for conducting path analyses. To control for multiple testing, we corrected for false discovery rate (FDR; Benjamini and Hochberg 1995) when necessary.

## Results

Urban males had higher positive scores for the relative warp 3 (Fig. 1c; $\chi^2 = 10.32$, d.f. = 1, $P = 0.001$, after FDR correction: $P = 0.006$). Moreover, urban males had a higher flight endurance (Fig. 2a; $\chi^2 = 32.73$, d.f. = 1, $P < 0.001$, after FDR correction: $P < 0.001$). None of the other measured traits differed significantly between urban and rural individuals (Fig. 1 and 2; all $P > 0.20$), neither did urban-rural differences depend on the mating status (Urbanisation level × Mating status: all $P > 0.12$).

The *a priori* path model where urban and rural populations were combined (Fig. 3a) was strongly supported by the data ($C_{16} = 7.19$, $P = 0.969$). Males with a lower fat content ($P = 0.015$), higher scores for relative warp 1 (slender wings, Fig. S3; $P = 0.017$), and higher wing loading scores ($P = 0.007$) had a higher flight endurance. Moreover, a significant negative quadratic effect of centroid size ($P = 0.016$) indicated that males with intermediate sized wings had higher flight endurance. Flight speed was not influenced by any measured physiological or morphological trait (all $P > 0.20$). Males with a lower wing loading ($P = 0.014$) had a higher mating success. Furthermore, a significant positive quadratic effect of relative warp 2 ($P = 0.016$) indicated that individuals with extreme high (short and broad wings) or low scores (long and slender wings) for relative warp 2 had a higher mating success (see also Fig. S3).

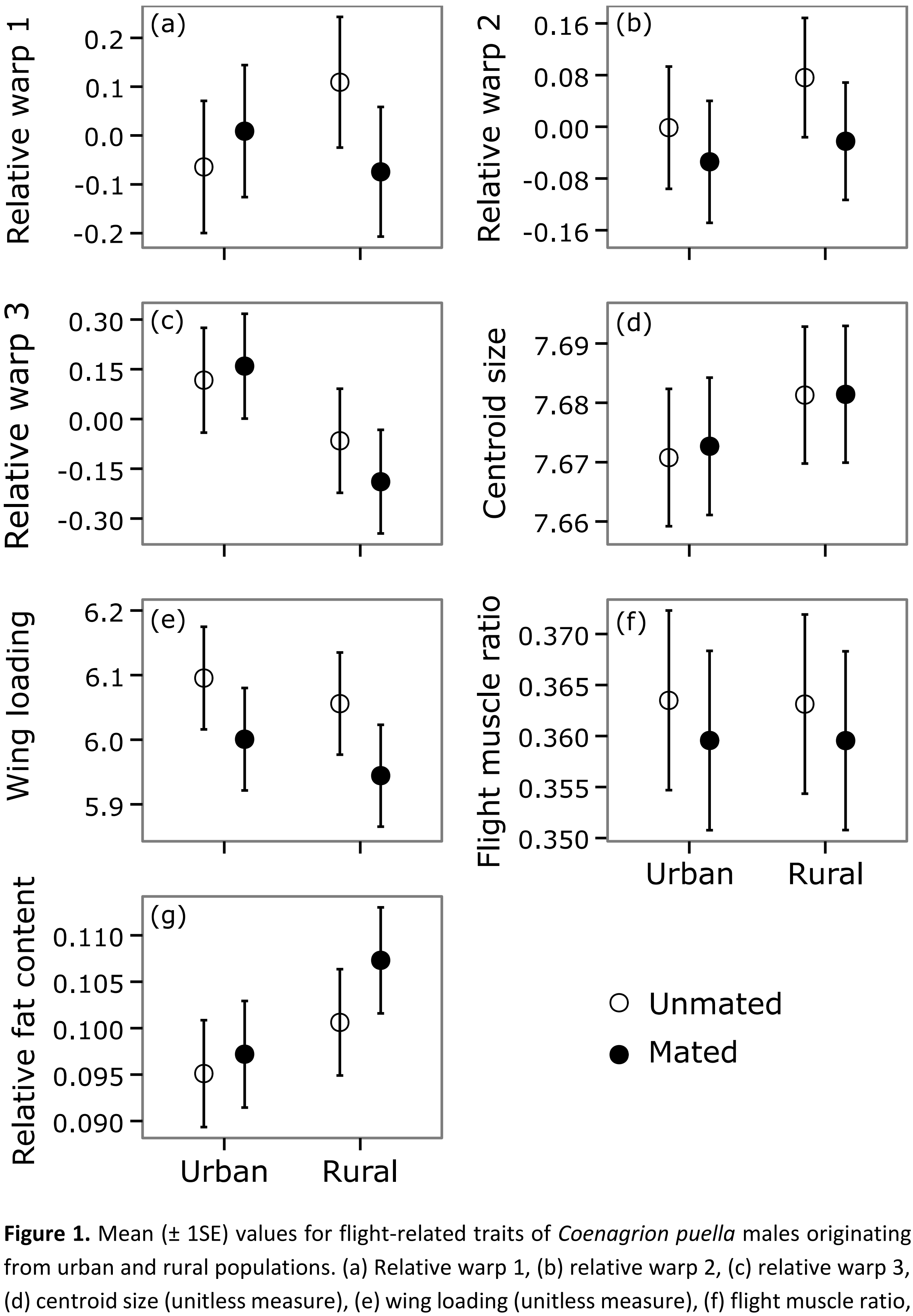

**Figure 1.** Mean (± 1SE) values for flight-related traits of *Coenagrion puella* males originating from urban and rural populations. (a) Relative warp 1, (b) relative warp 2, (c) relative warp 3, (d) centroid size (unitless measure), (e) wing loading (unitless measure), (f) flight muscle ratio, and (g) relative fat content. Given are least-squares means.

When we ran the *a priori* path model separately for urban and rural males (Fig. 3b), the goodness-of-fit of the path models slightly increased (Urban: $C_{16}$ = 7.75, $P$ = 0.956; Rural: $C_{16}$ = 8.49, $P$ = 0.933), suggesting the covariation patterns differed between urban and rural males. The path model for urban males showed that higher values for relative warp 1 ($P$ = 0.028) and wing loading ($P$ = 0.019) were positively associated with flight endurance. As in the combined path model, flight endurance decreased with increasing fat content for urban males (linear effect: $P$ = 0.009). Moreover, this decrease had a quadratic component ($P$ = 0.014). A significant negative quadratic effect of centroid size ($P$ = 0.026) indicated that males with intermediate sized wings had a higher flight endurance. Flight speed of urban males was not influenced by any physiological or morphological trait (all $P > 0.13$). A longer flight endurance (one-sided $P$ = 0.0305) increased the mating success of urban males. Mating success was affected by the quadratic term of relative warp 2 ($P$ = 0.033), indicating that individuals with short and broad wings or long and slender wings had a higher mating success. The path model for rural males, on the other hand, revealed that no variable had a significant effect on flight performance. Instead, we found links to mating success that were not present in the path model for urban males (Fig. 3): males with lower values for wing loading had higher mating success ($P$ = 0.013), whereas a significant negative quadratic effect of centroid size ($P$ = 0.035) indicated that males with intermediate centroid sizes had increased mating success.

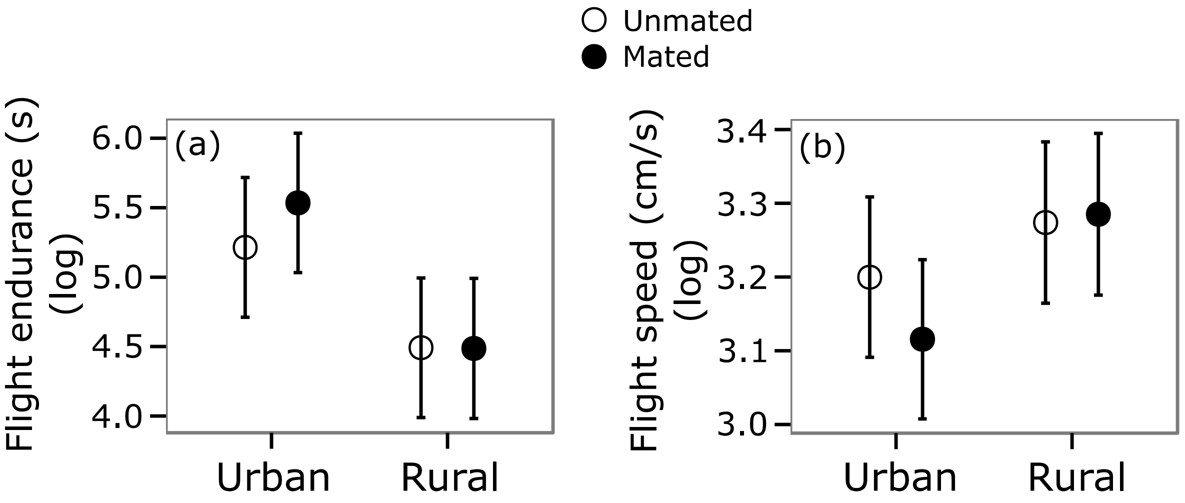

**Figure 2.** Mean (± 1SE) values for flight performance traits of *Coenagrion puella* males originating from urban and rural populations. (a) Flight endurance, measured as total flight duration, and (b) flight speed. Given are least-squares means.

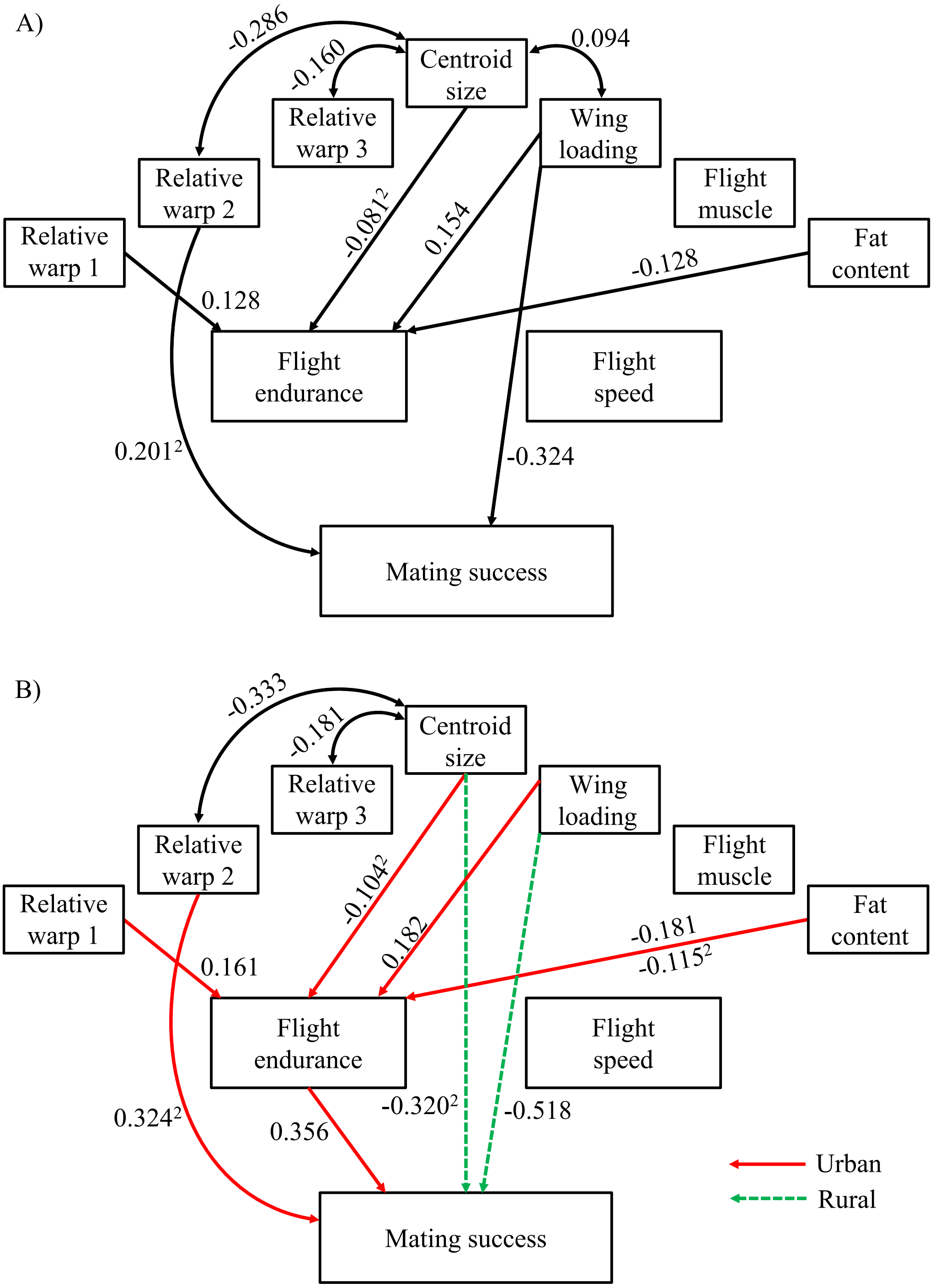

**Figure 3.** Path diagrams depicting the relationships between morphological and physiological traits, flight performance and mating success in urban and rural populations of the damselfly *Coenagrion puella*. Shown are path diagrams of A) the 'combined model', where combined data of urban and rural populations were used, and B) separate models for urban and rural populations. Double-headed arrows indicate correlations between traits. Path coefficients are given next to the arrows (see text and Table S2 for details). To increase clarity, only significant ($P < 0.05$) paths are shown. Paths with quadratic terms are indicated with $^{2}$.

Direct comparison confirmed several path coefficients to differ significantly between urban and rural models: the path going from the quadratic term of fat content ($t = 4.85$, df = 32, $P < 0.001$) to flight endurance, and paths from flight endurance ($t = 2.68$, df = 32, $P = 0.011$),the quadratic term of relative warp 2 ($t = 2.75$, df = 32, $P = 0.009$), and the quadratic term of centroid size ($t = 3.92$, df = 32, $P < 0.001$ ) to mating success. Significance of the differences did not change after controlling for FDR (Table S3). This confirmed that flight endurance was only under sexual selection in urban populations.

## Discussion

As expected, urban damselfly populations were differentiated from rural populations by having a higher flight endurance. Moreover, urban individuals differed in their wing shape, as captured by higher scores for relative warp 3. Also the covariation patterns and causal relationships among the phenotypic traits, performance and mating success differed between urban and rural populations. Notably, in line with our prediction, the higher flight performance of urban males was reinforced by sexual selection. We discuss these findings in terms of potential adaptations to urban habitats.

### *Differentiation in performance and flight-related traits between urban and rural populations*

A key novelty of our study was the support for our prediction of a higher flight endurance in urban damselflies. This is in line with a recent inter-specific study conducted in the same location (i.e. Flanders, Belgium), reporting that urban communities of carabid beetles consisted of species with better dispersal capacities compared to rural communities (Piano et al. 2017). While few intra-specific studies found indications of a better locomotor phenotype in urban populations (San Martin y Gomez and Van Dyck 2012; Schoville et al. 2013), this was not general (Evans et al. 2011), and no study directly compared flight performance between urban and rural populations. The latter is important as assumed phenotypic proxies for locomotor ability are not always empirically confirmed (e.g. Hanski et al. 2002). A higher flight endurance was expected given that urban ponds need to be colonised from source populations in rural areas (e.g. Evans et al. 2009) and habitats are more fragmented in urban areas (e.g. Luck and Wo 2002; Cane et al. 2006). Given these characteristics of urban habitats, the selection for a better locomotor ability in more isolated urban populations resembles the selection forces experienced at invasion fronts and in fragmented landscapes. High dispersal abilities at invasion fronts are predicted by spatial sorting: the better dispersers will be the ones colonising new habitats at the front, and assortative mating between fast-dispersing individuals at invasion fronts results in further increase in dispersal ability (Shine et al. 2011). Similarly, fragmented landscapes are expected to drive increased locomotor performance, facilitating easier movement between isolated habitat patches (Van Dyck and Matthysen 1999; Dover and Settele 2009). The few studies that directly compared locomotor ability in these related contexts mostly were consistent with a higher locomotor ability at expansion fronts and in populations located in more fragmented landscapes. Indeed, cane toads evolved a higher locomotor ability at the invasion front (Shine 2012), and flight endurance increased at the range expansion front in the damselfly *Coenagrion scitulum* (Swaegers et al. 2015). Further, the butterfly *Pieris brassicae* showed a higher flight performance with increasing landscape fragmentation (Ducatez et al. 2013). As we worked with field-caught individuals, besides spatial sorting also plastic responses related to higher water temperatures in urban ponds may have been at work (for the study region, Brans et al. 2018). Yet, we recently showed that larvae of the damselfly *Ischnura elegans* reared at higher temperatures showed lower flight endurance in the adult stage (Arambourou et al. 2017), making our finding of a higher flight endurance in the urban populations occupying warmer ponds conservative.

Changes in flight-related morphology associated with colonisation events and landscape fragmentation is common in flying insects, and is suggested to facilitate dispersal ability (Van Dyck and Matthysen 1999; Dover and Settele 2009; Hill et al. 2011). The direction of reported trait changes, however, is not consistent; especially so for wing size and shape. Longer and narrower wings, i.e. higher 'wing aspect ratios', have been reported for damselfly populations at range expansion fronts (*Calopteryx splendens*: Hassall et al. 2009) and occupying fragmented habitats (*C. maculata*: Taylor and Merriam 1995). Studies with the speckled wood butterfly *Pararge aegeria* reported higher (Hughes et al. 2007) or lower wing aspect ratios (Hill et al. 1999) at edge populations compared to the core populations, whereas another study revealed no relationship between landscape fragmentation and wing aspect ratio in the same species (Merckx and Van Dyck 2006). Moreover, larger wings in urban compared to rural populations have been documented in insects (the grasshopper

*Chorthippus brunneus*: San Martin y Gomez and Van Dyck 2012, the butterfly *Pieris rapae*: Schoville et al. 2013), and was suggested to result from selection for increased mobility. On the other hand, decreasing patch connectivity was associated with decreasing wing length in the blue-winged grasshopper *Oedipoda caerulescens*, and was explained by lower investment in dispersal ability due to increasing cost of dispersal (Heidinger et al. 2010).

Given these conflicting patterns in flight-related morphology associated with colonisation events and landscape fragmentation, it may not be surprising that we have not found substantial differences in wing size and shape between urban and rural populations in the current study. Indeed, wing length (measured as centroid size) was not significantly different between urban and rural individuals. Further, the wing shape parameter relative warp 2, which can be considered analogous to wing aspect ratio (negative scores of relative warp 2 imply long and narrow wings; i.e. high wing aspect ratio), did not differ between urban and rural males. The only signal of urban-rural differentiation we detected was a higher score for relative warp 3 in urban compared to rural populations. However, this wing shape variable explained only ca. 9% of the total wing shape variation, hence only contributed little to the higher flight endurance in urban males. Furthermore, the subtle shape changes associated with relative warp 3, which are mainly focused on the wing apex (Fig. S3) make the interpretation of this wing shape variation difficult.

### *Differentiation in sexual selection on flight performance between urban and rural populations*

In agreement with theory and previous empirical findings, we found positive sexual selection for higher flight endurance, yet only in the urban populations. For damselflies, this pattern has been demonstrated before, both directly by contrasting flight performance of mated and unmated males (Therry et al. 2014b; Gyulavári et al. 2017; for the study species: Gyulavári et al. 2014), as well as indirectly by showing sexual selection on flight-related traits (e.g. De Block and Stoks 2007). Increased flight performance in scrambling mating systems is expected, as males with a higher mobility will encounter, hence mate with, more females (Kelly et al. 2008). As suggested (Husak and Fox 2008) and empirically shown (Gyulavári et al. 2014; Therry et al. 2014b), flight speed was not under sexual selection, confirming that flight speed is less important for scramble competitors. As expected, flight endurance was under sexual selection only in urban, and not in rural males. This somewhat matches the absence of sexual selection on maximum flight distance in a rural population of *Lestes sponsa* damselflies (Outomuro et al. 2016). The differentiation between population types in selection on flight endurance can be explained by the associated differentiation in mean flight endurance. Indeed, population differences in mean phenotypic traits may affect the direction and strength of selection, as populations will then span different parts of the species' fitness function (Endler 1986). For example, Steele et al. (2011) showed that temporal variation in mean body size of the damselfly *Enallagma aspersum* resulted in changes in the experienced fitness function at a given moment in time. For the present study, the shape of the overall fitness function across all populations reveals that while lower flight endurance is not under selection, flight endurance generates a mating advantage at higher endurance values (as present in the urban males). This confirms the shape of the fitness function for flight endurance as was previously reported for the study species (Fig. 1b in Gyulavári et al. 2014). We discuss the urban-rural differentiation in sexual selection on flight-related traits, and the differentiation in covariation patterns with flight performance in more detail in the Appendix.

Sexual selection is thought to accelerate adaptation to anthropogenic environments (Candolin and Heuschele 2008), and at the same time anthropogenic environments have been shown to shape sexual selection (e.g. Candolin et al. 2007; Lane et al. 2011). Despite these insights, we have an extremely limited understanding of how urbanisation, and even more general, habitat fragmentation can alter sexual selection (Murphy et al. 2016). A notable exception in vertebrates is the divergence in sexually selected traits (male genitalia size and dorsal-fin coloration) due to habitat fragmentation in *Gambusia* fish (Heinen-Kay et al. 2014; Giery et al. 2015), however in this system the main factors driving this divergence was not fragmentation *per se,* but altered community composition and water chemistry. We also have poor knowledge whether sexual selection is aligned with natural selection and thereby reinforces natural selection in driving adaptive divergence between populations (Hendry 2016). Our results provide the first evidence for a higher flight endurance in urban populations that was moreover associated with sexual selection for increased flight endurance in urban populations. Given that the increased flight endurance at edge populations of a related *Coenagrion* damselfly has been associated with genotypic differences (Swaegers et al. 2015), we might expect evolutionary divergence in flight performance in the derived urban damselfly populations. Together, this highlights the intriguing pattern that while urbanisation shapes flight endurance and the associated sexual selection on endurance, the resulting sexual selection pattern reinforces the higher flight endurance in urban populations. Our results thereby provide unique proof in a single study system of the interplay between sexual selection and adaptation to anthropogenic environments (Candolin and Heuschele 2008).

## Acknowledgements

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## Appendix

**Table S1.** Coordinates and sampling information of the three urban and three rural populations of *Coenagrion puella* studied. Flight performance was quantified for only a subset of males.

| Population | Urbanisation level | Sampling period | Total number of males | | Total number of males in flight test | |
|---|---|---|---|---|---|---|
| | | | unmated | mated | unmated | mated |
| Bierbeek | Rural | 14-17.07.2013 | 47 | 50 | 25 | 25 |
| Bornem | Rural | 24-25.07.2013 | 47 | 50 | 29 | 29 |
| Houwaart | Rural | 15-19.07.2013 | 52 | 51 | 32 | 26 |
| Leuven | Urban | 08-17.07.2013 | 49 | 53 | 28 | 29 |
| Mechelen | Urban | 16-29.07.2013 | 40 | 40 | 27 | 29 |
| Oudenaarde | Urban | 13-28.07.2013 | 49 | 48 | 29 | 30 |

**Table S2.** Standardized path coefficients of the 'combined model' (where combined data of urban and rural males were used), the 'urban model' (only including urban males), and the 'rural model' (only including rural males). Paths with quadratic terms are indicated with $^2$. Significant ($P <$ 0.05) paths are given in bold. Note that we report one-sided *P* values for the "Mating success – flight endurance" path.

| Path | | Combined model | | | Urban model | | | Rural model | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Response | Predictor | Coefficient | SE | *P* | Coefficient | SE | *P* | Coefficient | SE | *P* |
| Flight endurance | Relative fat content | **-0.128** | **0.052** | **0.015** | **-0.181** | **0.068** | **0.009** | -0.133 | 0.084 | 0.138 |
| | Relative fat content$^2$ | -0.029 | 0.034 | 0.404 | **-0.115** | **0.046** | **0.014** | 0.05 | 0.055 | 0.37 |
| | Relative warp 1 | **0.128** | **0.053** | **0.017** | **0.161** | **0.071** | **0.028** | 0.101 | 0.079 | 0.207 |
| | Relative warp 2 | -0.001 | 0.052 | 0.987 | 0.066 | 0.072 | 0.36 | -0.067 | 0.076 | 0.391 |
| | Relative warp 3 | 0.035 | 0.054 | 0.517 | 0.156 | 0.081 | 0.055 | -0.061 | 0.075 | 0.423 |
| | Wing loading | **0.154** | **0.056** | **0.007** | **0.182** | **0.076** | **0.019** | 0.173 | 0.083 | 0.051 |
| | Centroid size | -0.017 | 0.057 | 0.771 | 0.013 | 0.079 | 0.869 | -0.004 | 0.085 | 0.964 |
| | Centroid size$^2$ | **-0.081** | **0.033** | **0.016** | **-0.104** | **0.046** | **0.026** | -0.055 | 0.049 | 0.274 |
| Flight speed | Flight muscle ratio | 0.028 | 0.054 | 0.611 | 0.017 | 0.075 | 0.827 | 0.061 | 0.082 | 0.466 |
| | Relative fat content | 0.047 | 0.055 | 0.407 | -0.052 | 0.075 | 0.484 | 0.145 | 0.084 | 0.107 |
| | Relative warp 1 | -0.072 | 0.057 | 0.211 | -0.12 | 0.079 | 0.133 | -0.006 | 0.086 | 0.945 |
| | Relative warp 2 | 0.006 | 0.053 | 0.913 | -0.013 | 0.075 | 0.86 | 0.004 | 0.078 | 0.957 |
| | Relative warp 3 | -0.008 | 0.058 | 0.893 | -0.03 | 0.087 | 0.732 | 0.053 | 0.081 | 0.514 |
| | Wing loading | -0.077 | 0.06 | 0.204 | -0.067 | 0.083 | 0.419 | -0.123 | 0.091 | 0.198 |

**Table S2.** (continued)

| Path | | Combined model | | | Urban model | | | Rural model | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Response | Predictor | Coefficient | SE | *P* | Coefficient | SE | *P* | Coefficient | SE | *P* |
| Mating success | Flight endurance | 0.204 | 0.125 | 0.051 | **0.356** | **0.19** | **0.030** | 0.02 | 0.187 | 0.458 |
| | Flight speed | -0.065 | 0.118 | 0.58 | -0.114 | 0.168 | 0.496 | -0.014 | 0.181 | 0.936 |
| | Flight muscle ratio | -0.214 | 0.117 | 0.068 | -0.21 | 0.165 | 0.204 | -0.293 | 0.184 | 0.111 |
| | Relative fat content | 0.163 | 0.119 | 0.17 | 0.076 | 0.163 | 0.641 | 0.275 | 0.191 | 0.149 |
| | Relative warp 1 | -0.141 | 0.123 | 0.252 | -0.129 | 0.171 | 0.452 | -0.173 | 0.189 | 0.359 |
| | Relative warp 2 | -0.131 | 0.124 | 0.293 | -0.162 | 0.184 | 0.377 | -0.105 | 0.184 | 0.568 |
| | Relative warp $2^2$ | **0.201** | **0.083** | **0.016** | **0.324** | **0.152** | **0.033** | 0.096 | 0.104 | 0.355 |
| | Relative warp 3 | -0.002 | 0.126 | 0.988 | 0.073 | 0.19 | 0.701 | -0.01 | 0.182 | 0.955 |
| | Wing loading | **-0.324** | **0.131** | **0.014** | -0.27 | 0.183 | 0.14 | **-0.518** | **0.209** | **0.013** |
| | Centroid size | 0.03 | 0.138 | 0.829 | 0.09 | 0.202 | 0.656 | -0.039 | 0.211 | 0.855 |
| | Centroid size$^2$ | -0.104 | 0.089 | 0.245 | 0.029 | 0.136 | 0.829 | **-0.32** | **0.152** | **0.035** |

**Table S3.** Direct pairwise comparison of path coefficients derived from the 'urban model' (including only urban males) and the 'rural model' (including only rural males). Paths with quadratic terms are indicated with $^2$. Paths that differed significantly ($P < 0.05$) are indicated in bold.

| Path | | Difference of path coefficient (Rural – Urban) | Pooled SE | *t* | df | *P* | *P* (after FDR correction) |
|---|---|---|---|---|---|---|---|
| Response | Predictor | | | | | | |
| Endurance | Relative fat content | 0.048 | 0.052 | 0.923 | 32 | 0.363 | 0.408 |
| | Relative fat content$^2$ | **0.165** | **0.034** | **4.853** | 32 | **< 0.001** | **< 0.001** |
| | Relative warp 1 | -0.06 | 0.053 | 1.132 | 32 | 0.266 | 0.342 |
| | Wing loading | -0.009 | 0.056 | 0.161 | 32 | 0.873 | 0.873 |
| | Centroid size$^2$ | 0.049 | 0.033 | 1.485 | 32 | 0.147 | 0.221 |
| Mating | Endurance | **-0.336** | **0.125** | **2.688** | 32 | **0.011** | **0.025** |
| | Relative warp 2$^2$ | **-0.228** | **0.083** | **2.747** | 32 | **0.010** | **0.025** |
| | Wing loading | -0.248 | 0.131 | 1.893 | 32 | 0.067 | 0.121 |
| | Centroid size$^2$ | **-0.349** | **0.089** | **3.921** | 32 | **< 0.001** | **0.002** |

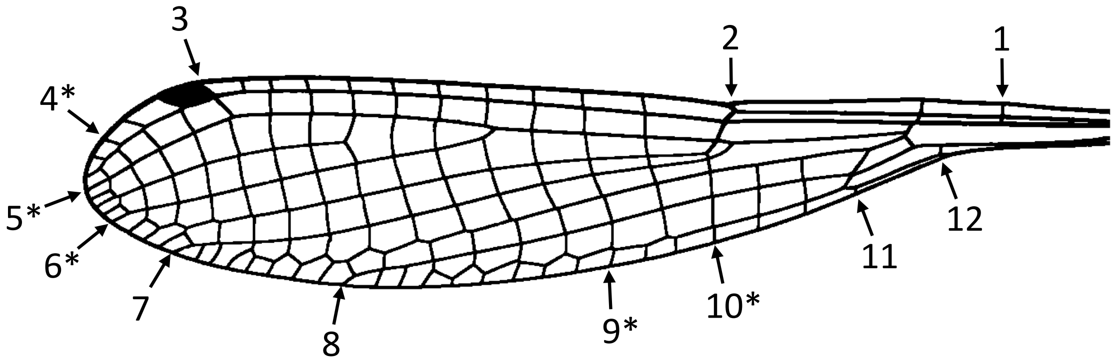

**Figure S1.** Location of landmarks used to describe wing shape in *Coenagrion puella* males. Numbers with asterisks correspond to semi-landmarks.

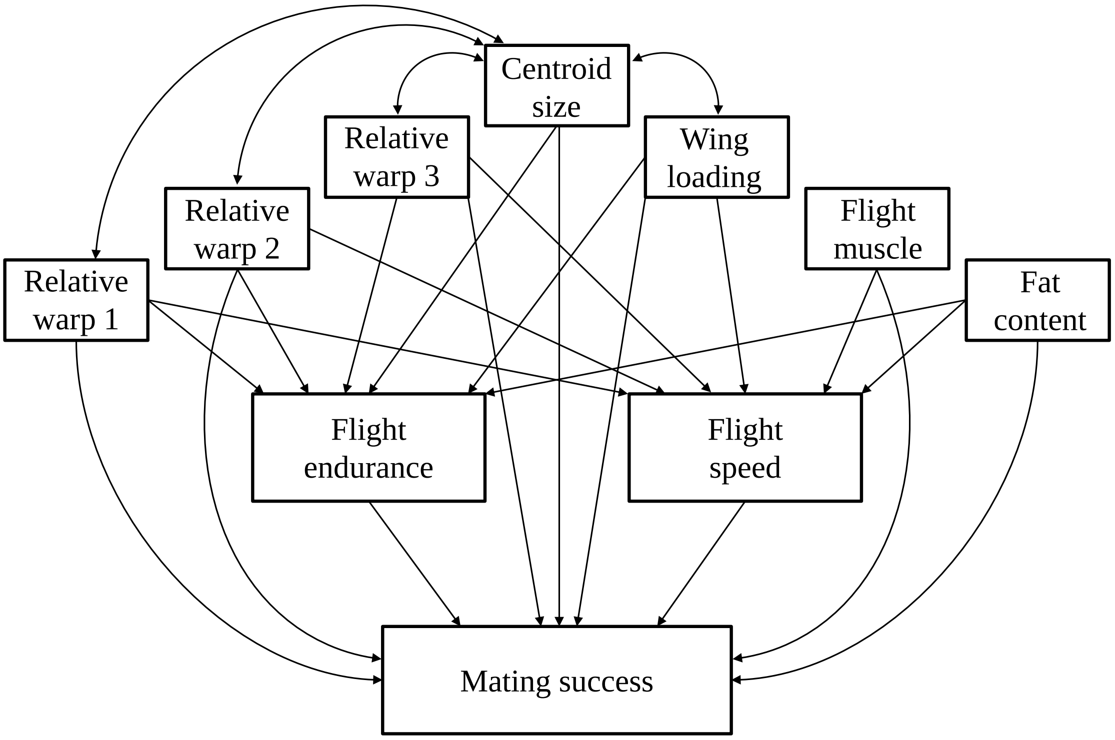

**Figure S2.** *A priori* path diagram, depicting the hypothesized relationships between morphological and physiological traits, flight performance and mating success in damselflies. In addition to linear terms, quadratic terms of relative fat content (with flight endurance as response variable), relative warp 2 (with mating success as response variable), and centroid size (with both flight endurance and mating success as response variable) were included to the *a priori* path model. Double-headed arrows indicate correlations between traits.

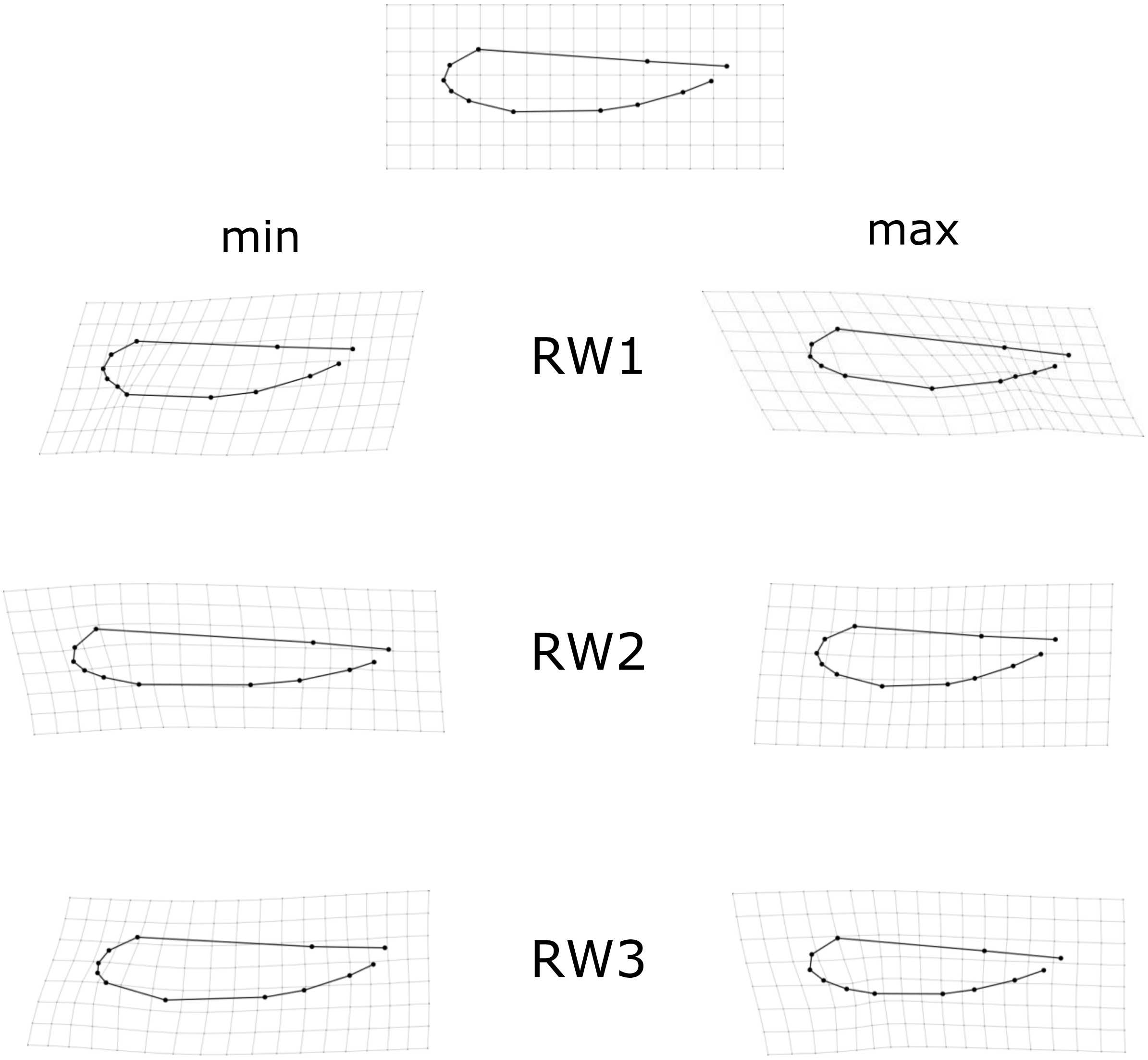

**Figure S3.** Wing shape variation in *Coenagrion puella* males, illustrated using thin-plate spline transformation grids. The first three relative warps (RW1-3) explained 81.4% of the wing shape variation. Negative (left panel) and positive (right panel) extremes for the three relative warps are shown. The most upper panel represents the mean wing shape. Transformation grids for RW2 and RW3 have been amplified (×3) for better illustration.

## Additional discussion

### *Differentiation in covariation patterns between urban and rural populations*

Flight performance was associated with various phenotypic traits, but the relationship strongly depended on the urbanisation level. Urban males with slender wings (positive scores for relative warp 1) had increased flight endurance. The mechanistic advantage of this wing type in terms of flight endurance cannot easily be linked to flight biomechanical studies as these typically measure wing aspect ratios instead of independent measures of wing length and width. Wings with intermediate centroid sizes resulted in a higher flight endurance, but only in urban males. This somewhat matches the absence of an effect of wing size (linear or quadratic) on maximum flight distance in a rural population of *Lestes sponsa* damselflies (Outomuro et al. 2016). The advantage of intermediate wing size in terms of flight endurance in urban males is not clear Wing loading was positively correlated with flight endurance in urban populations. Although this is in disagreement with the notion of the energetically more efficient low wing loading (Angelo and Jr. 1984), it has been reported in previous studies (e.g. Berwaerts et al. 2002). It is possible that all sampled populations from urban areas occupy habitats with abundant resources, compensating for the increased flight cost of higher wing loadings (Bartholomew and Casey 1978). Contrary to our expectation of a positive correlation of flight endurance and fat content (flight duration in insects depends on the amount of energy stores, Kaufmann et al. 2013), we found that fat content was associated with lower flight endurance in urban individuals, whereas there was no relationship in rural individuals (see also Gyulavári et al. 2017). Complex and opposite relationships between flight endurance and fat content may be partly explained by high fat contents allowing long flights, but at the same time long flights reducing the fat content (Arrese and Soulages 2010).

### *Differentiation in sexual selection on flight-related traits between urban and rural populations*

Several flight-related traits were identified as targets of sexual selection and again, strongly depending on the population type. In urban populations, four traits (relative warp 1, centroid size, wing loading and relative fat content) were under indirect sexual selection as they contributed to mating success through their effect on flight endurance (discussed above). In addition, relative warp 2 directly shaped mating success. This took the form of disruptive sexual selection, indicating higher mating success for males with either long and narrow (high wing aspect ratio), or short and broad wings (low wing aspect ratio). This could indicate that these extreme wing shapes either contribute to an unmeasured aspect of flight performance which in turn increases mating success, or provide an advantage for mating success for other reasons. Instead, in rural populations we only found negative directional sexual selection for wing loading not mediated through flight endurance. Larger wings relative to body mass may indeed be advantageous for acquiring mates in scrambling mating systems, as it is energetically less costly (Angelo and Jr. 1984), hence allows allocating more time to searching females instead of food. Our study does not allow to explain why this would apply only to rural, and not urban males. Possibly, the higher air temperatures in urban areas (the so-called "urban heat island" effect: Arnfield 2003) create more favourable flight conditions, thereby reducing costs of long flight for urban males. Similarly, the negative directional sexual selection for wing loading in rural males could be a compensation for reduced wing beat frequency at the typically cooler temperatures of rural areas (e.g. Merckx et al. 2006). Finally, we detected stabilising sexual selection on wing size (also proxy for body size) only in rural populations. This pattern has previously been shown in damselflies (Stoks 2000, Thompson and Fincke 2002; Gyulavári et al. 2017). Interestingly, a study with the damselfly *Ischnura elegans* revealed that the selection on male body size can take almost any form, depending on the sampled population and the year of sampling (Gosden and Svensson 2008). The authors suggest this was driven by densities of the female colour morphs (which also occurs in the study species), and female body sizes. We did not find any urbanisation-related difference in female body size (unpublished data), yet we have no data on the density of female colour morphs from the sampled locations, which could potentially explain the spatial variation in selection on body size found in the present study.

# GENERAL DISCUSSION

In this chapter, I will first discuss the documented responses to each of the three urban-related stressors tested in this thesis project, with emphasis on potential signals of adaptations to urban environments. Next, I will focus on the stage- and sex-specific aspects of these responses, and evaluate my findings within the pace-of-life syndrome framework. After identifying some limitations of my study and key gaps for future research, I will discuss the contributions of my findings to the field of adaptations to urbanisation.

## *(i) Responses to temperature*

My findings do not support a scenario of thermal adaptation, whereby urban individuals are expected to perform better at higher temperatures compared to rural individuals. The lack of thermal adaptation was consistent when using several response variables as performance traits, including growth rate, survival (chapter 1 and 2), egg development rate, and size at hatching (chapter 1). This is in contrast with the few studies reporting higher heat tolerance in urban compared to rural populations (ants: Angilletta et al. 2007 and Diamond et al. 2017; waterflea: Brans et al. 2017b). Instead, I propose that the extended growing season in urban areas, an indirect effect of UHIs, is driving the here reported slower growth rates of urban damselflies along a range of temperatures. This vertical shift in TPCs, also called countergradient variation, represent a scenario where the environment and genotype influence the phenotype in opposite directions (Conover and Schultz 1995; Conover et al. 2009). Under natural conditions, individuals adapted to colder environments can have similar or lower performance compared to warm-adapted individuals, hence the genetic variation is concealed under natural conditions. When tested under common garden conditions, individuals originating from cold environments are released from the constraining effect of low temperature they experience in their natural environment, hence outperform warm-adapted individuals. A vertical shift in TPCs towards lower performance under common garden settings has been reported before for populations living in warmer, hence less time-constrained environments (fish from low vs high latitudes: Yamahira and Conover 2002; frogs from shaded vs unshaded ponds: Richter-Boix et al. 2015), and once in the context of urbanisation (McLean et al. 2005). Yet, the latter study with terrestrial fungi did not find a consistent pattern across the tested isolates (McLean et al. 2005).

Several reasons may underlie the (apparent) differences with the three studies showing a higher heat tolerance in urban populations of ants (Angilletta et al. 2007; Diamond et al. 2017) and water fleas (Brans et al. 2017b). First, the time constraints that drive the rapid growth before winter in rural larvae may be stronger, and the cost of rapid growth considerable driving a rapid reduction in growth rate in the more time-relaxed urban populations of damselflies. This is likely so when comparing with water fleas that have many clonal generations per year, making rural populations less time constrained (Blanckenhorn and Demont 2004). Second, the studies on ants and water fleas did not consider growth rate, whereas thermal performance curves may differ between traits (Sinclair et al. 2016). Moreover, as the other studies did not test performance across a range of temperatures (only looked at the upper temperature where performance was reduced to zero), it cannot be fully excluded that TPCs actually show similar urban-rural divergence at lower temperatures. Finally, a higher heat tolerance has been shown to trade off with the chronic ability to deal with mild heat waves (Rezende et al. 2014), and damselfly larvae may be differentially trading off the ability to withstand the rare strong vs the more common mild heat waves.

I also did not find a signal of thermal adaptation with regard to other differences in the thermal regimes between urban and rural ponds. It is well known that effects of temperature fluctuations and extreme temperatures on organismal traits can differ in magnitude and direction from the effects of mild, constant increases in temperatures (Colinet et al. 2015). This is supported by findings that TPCs can be sensitive to temperature variations experienced in natural conditions (e.g. Latimer et al 2011; Richter-Boix et al. 2015). Therefore, even though I did not detect thermal adaptation to the relatively high temperatures in urban habitats, other characteristics of urban thermal regimes, such as extreme temperatures and increased temperature variations, may be driving TPC differentiations between urban and rural populations. For genotypes adapted to high temperature variations, a broader thermal breadth (i.e. wider TPCs) is theoretically predicted (Lynch and Gabriel 1987; Gilchrist 1995), yet growth rate TPCs for urban and rural larvae did not differ in their width (chapter 1).

### *(ii) Responses to pesticide exposure*

In chapters 2 and 3, I tested whether the urbanisation level of the individuals determined their behavioural responses to sublethal doses of pesticide exposure. When exposed to the pesticide chlorpyrifos, rural larvae decreased their activity, whereas urban larvae instead increased their activity, consistent with a scenario of local adaptation to higher contaminant levels. Yet, when exposed to esfenvalerate, a synthetic pyrethroid pesticide, I found nearly the opposite pattern: activity levels were reduced in urban larvae, but not in rural larvae. Several factors may be at play for these contrasting findings. First, the mode-of-action of the two pesticide classes differ from each other, chlorpyrifos being a acetylcholinesterase inhibitor, and esfenvalerate a sodium channel modulator. Second, the two pesticides strongly differ in their physico-chemical properties. Both the mode-of-action and the physico-chemical properties may dictate the effect of a pesticide on organismal traits (Guénard et al. 2014; Halstead et al. 2015). Finally, the different exposure duration of the chlorpyrifos and esfenvalerate treatments (15 days vs 14 h, respectively) may have driven the nearly opposite patterns. Possibly, urban larvae only increase their activity and food intake in the short term to overcome short pesticide pulses, but are not able to do so under more chronic pesticide exposure. This would match the well-known hormetic responses seen in ecotoxicology (Cedergreen et al. 2005; Costantini et al. 2010).

The level of urbanisation from which the damselflies originated did not play a role in the repeatability of the behaviours. Based on the feedback loop between the toxicity of the contaminant and the behavioural type of the individual (Montiglio and Royauté, 2014; described in detail in the *General introduction*), contaminant exposure is predicted to either increase (Tosetto et al. 2017; White and Briffa, 2017) or decrease behavioural repeatability (Kolok et al. 1998; Dzieweczynski, 2011; Royauté et al. 2015; Dzieweczynski et al. 2016). Note that I did not separately model the two variance components used for repeatability calculations, i.e. between- and within-individual variation. Therefore, I cannot ignore the possibility that either (or both) of the variance components actually did change with the pesticide treatment (as in Royauté et al. 2015), which nevertheless resulted in an overall no change in repeatability. Unlike repeatability, the effect of the pesticide exposure on the behavioural correlations differed strongly between urban and rural larvae. The already-present correlation between activity and boldness in the urban larvae did not change with exposure to the pesticide, whereas the same correlation in rural larvae only occurred after being exposed to the pesticide. Given that behavioural correlations have often been documented to develop under stressful conditions (e.g. Bell 2005; Dingemanse et al. 2007; Snekser et al. 2009), the finding that only urban larvae exhibited a (pre-pesticide exposure) behavioural correlation may suggest that urban habitats in general impose stronger selection pressures on damselfly larvae compared to rural habitats. It should be noted that the frequent finding of an absence of behavioural correlations in urban bird populations (Evans et al. 2010; Scales et al. 2011; Riyahi et al. 2017) does not contrast with my findings as for birds, cities are often considered the more favourable habitats compared to rural habitats (less food shortage and less predator stress).

Notably, when testing for the effects of exposure to sublethal doses to a pesticide (chapters 4 and 5), I found that the urbanisation level did not determine how the pesticide effect carries over to later life stages. Given that damselflies actually do suffer from pesticide exposure during the larval stage in terms of adult fitness (e.g. reduced lifetime mating success in females: chapter 4), any urbanisation-driven adaptive response to pesticide exposure (i.e. behavioural responses: chapter 2 and 3) seems to be ineffective for reducing carry-over effects. This further highlights the need for a life cycle approach in ecotoxicology (Debecker et al 2017).

### ***(iii) Responses to habitat fragmentation***

Given that dispersal in semi-aquatic insects is in the adult stage, I predicted the selection pressure of increased fragmentation and isolation of urban habitats to act on the adult damselflies. I interpret the higher flight performance of urban damselflies (chapter 6) to be a result of spatial sorting (Shine et al. 2011), whereby only the best dispersers, in this case best flyers, are able to reach the more fragmented and isolated urban ponds.

Even though I intensely studied larval responses to a range of temperatures, I did not test for carry-over effects of the UHI on adult flight performance. In a recent study, the dragonfly *Erythemis collocata* was suggested to have a reduced flight performance when larvae had undergone mild warming, as these emerged with smaller wings (McCauley et al.

2018). Similarly, the damselfly *Ischnura elegans* had reduced flight endurance when exposed to warming during larval development (heat wave: Arambourou et al. 2017; mild warming: Tüzün et al. in press). Given the warmer water temperatures in urban areas, this finding may seem to contradict with the here documented higher flight endurance of the urban damselflies (chapter 6). Yet, the referred studies identified altered wing shape (Arambourou et al. 2017) or reduced flight muscle mass (Tüzün et al. in press) as mediators for the reduced performance, while the damselflies collected from urban ponds (chapter 6) did not differ from rural individuals in terms of their flight muscle mass. Urban males did have a slightly altered wing shape (i.e. relative warp 3), which was, however, not associated with the higher flight performance. These findings support our interpretation of spatial sorting being the main driver of the better flight performance of urban damselflies, and suggests that distinct abiotic conditions between urban and rural habitats, such as the UHI, experienced early in life did not play a major role in shaping flight performance. It should be noted that the evolutionarily-fixed slower growth rates of urban populations (chapter 1), here interpreted as a response to the extended growing season (driven by the UHI effect), may pose an alternative or complementary mechanism for their higher flight endurance. When compared to rural populations, the less time-stressed larvae of urban populations apparently do not need to invest in accelerated somatic growth, which may spare them from experiencing costs later in the adult stage due to trade-offs (e.g. Dmitriew 2011), potentially in the form of reduced flight ability. Costs of rapid growth in terms of reduced mobility have indeed been demonstrated in many taxa (e.g. in fish: Álvarez and Metcalfe 2005; Lee et al. 2016). One other related reason may reconcile the cited studies and my findings. The cited studies all showed a negative effect of warming in rural populations, generating the new hypothesis that larval warming only negatively affects flight ability in rural populations in case of thermal adaptation in the urban populations.

This urbanisation-driven change in flight performance had a crucial consequence for mating success. Better flying males were more likely to acquire a mate compared to their competitors, yet this was only true for urban, and not rural males. This finding provides unique evidence for how urbanisation-driven changes in dispersal capacity can influence sexual selection regimes. Human-altered environments can shape sexual selection (e.g.

Candolin et al. 2007; Lane et al. 2011), while sexual selection itself can accelerate (or hinder) adaptation to human-altered environments (Candolin and Heuschele 2008). My findings suggest that urbanisation-driven habitat fragmentation can indeed facilitate such processes.

### Stage- and sex-specific responses to urban-stressors

Organisms often face distinct environmental conditions along their ontogeny – especially so if they have complex life cycles, for example an aquatic larval stage and terrestrial adult stage as in the study species (Wilbur 1980; Moran 1994). This leads to the expectation of ontogeny-specific responses to selection pressures, including those caused by urban-related stressors. Further, age-dependent physiological changes can also contribute to such distinct responses across ontogeny (e.g. for thermal tolerance: Bowler and Terblanche 2008). Besides life stages, also sexes may be expected to differ in their response to urbanisation. Sexes indeed differ in selection pressures resulting in divergent life history trajectories and phenotypic trait values (Fairbairn et al. 2007). For example, physiological responses to urban-related disturbance was found to be sex-specific in the white-crowned sparrow *Zonotrichia leucophrys*, potentially due to differences in resources allocation (Bonier et al. 2007). Importantly, due to sex-specific differences in plasticity, ontogeny-dependent responses to stressors can be different for males and females (e.g. for temperature: Stillwell et al. 2009; Ketola et al. 2012; Knapp and Nedvěd 2013).

The outdoor common garden experiments, where I studied urban-related stress responses across the life cycle of the damselfly (chapters 4 and 5) are particularly useful to interpret stage- and sex-specific responses to urbanisation. Overall, while the urbanisation level played a crucial role for the way males responded to urban stressors, females responses did not depend on the urbanisation level; neither during metamorphosis, nor later as adults. For example, effects of time stress on mass at emergence and lifespan of males depended on the urbanisation level, but not for females. Studies with birds confirm the prevalence of sex-specific carry-over effects (López-Calderón et al. 2017; Masero et al. 2017; Saino et al. 2017). One reason may be that females are under strong fecundity selection to keep mass at emergence as high as possible (potentially trading it off against other traits). Moreover, female damselflies have a larger mass increase and a longer maturation time after emergence

(Stoks 2001), possibly allowing them to better compensate for stressors experienced in the larval stage.

Seasonal changes in environmental conditions, hence altered selection pressures, experienced within a given life stage may be another factor causing ontogeny-dependent differentiation in responses. Urban larvae had an overall slower growth rate compared to rural larvae only between days 30 and 50, and not during the first month (chapter 1) or in the final instar (chapter 2). It can be expected that damselfly larvae face the strongest time constraints when larvae try to reach a certain developmental stage before the onset of winter (corresponding to the period of day 30-50), potentially adding pressure to the already time-stressed rural larvae.

The animal personality literature has recently acknowledged sex-dependent differences in repeatability, personality and behavioural syndromes (Pruitt and Riechert 2009; Schuett and Dall 2009; Hedrick and Kortet 2012; Chapman et al. 2013; Fresneau et al. 2014; Michelangeli et al. 2016; Hämäläinen et al. 2018). Moreover, personality differences (especially for mating behaviours) between sexes is a central topic of sexual selection studies (Schuett et al. 2010). Yet, this was never tested under exposure of anthropogenic (urban-related) stressors. I found no sex-dependency in the pesticide effect on repeatability or behavioural syndrome structure (chapter 3), suggesting that in the study species, pesticide-induced changes in behavioural consistency and behavioural correlations are independent of the sex, at least in the studied larval stage.

## Pace-of-life syndrome in the context of urbanisation

While studies on urbanisation typically focused on single traits, traits often do not (co)vary independently, making a multitrait approach relevant when studying phenotypic differentiation along environmental gradients. The idea that suit of traits can covary in response to environmental conditions dates back to the early work on life history theory (Roff 1992; Stearns 1992). Trade-offs between resource allocation can create covariation patterns between life-history traits that align along a slow-fast continuum (Stearns 1983). The term pace-of-life was used to define the position of a given species or population along this continuum, which also acknowledged the integration of physiological traits into the suit of correlated traits, forming a so-called pace of life syndrome (POLS, Ricklefs and Wikelski 2002; Wikelski et al. 2003). Many studies convincingly showed that species and populations differentiated along latitudinal gradients largely in line with the POLS. The relatively recent concept of an extended POLS predicts suites of life-history, behavioural and physiological traits to be tightly linked, and align along a fast-slow continuum. For example, a fast growth and development is often associated with more active and risk-prone behaviours and physiological trait values such as a high metabolic rate (Careau et al. 2008; Réale et al 2010). While successful at the species level, evidence questions the generality of this concept at the level of populations and individuals (e.g. Debecker et al. 2016; Santostefano et al. 2017; Jablonszky et al. 2018), whereby a recent meta-analysis found little overall support for the extended POLS hypothesis (Royauté et al. 2018). This can often be due to a lack of integration of behavioural traits into the POLS (e.g. Niemelä et al. 2013), and might be related to the fact that the POLS is strongly dependent on the environmental context (e.g. Hille and Cooper 2015; Segev et al. 2017; Goulet et al. 2017; Michelangeli et al. 2018).

The urbanisation gradient represents a strong anthropogenically modified environmental gradient, resembling to some extent thermal latitudinal gradients. Not surprisingly, many studies on the effects of urbanisation on species traits report their findings within a POLS framework; especially so for bird studies. Results of a recent meta analysis with birds suggests a slower pace of life in urban populations, in this case high adult survival and smaller annual clutch size, which was suggested to be a result of increased food quantity, lower macroparasite abundance and/or lower risk of predation in cities (Sepp et al. 2018). Yet, many exceptions exist. Great tit populations in the city, for example, laid eggs earlier, had smaller clutches, and the offspring fledged in poorer condition compared to forest populations (Sprau et al. 2017), hinting at a faster pace of life of urban populations. Similarly, a study with great tits which included also behavioural traits reports a faster pace of life of urban populations, in this case higher breath rate and faster exploration (Charmantier et al. 2017), potentially a consequence of the novel and more stressful urban environment. These findings suggest that there is no general pattern emerging for the effects of urbanisation on the pace of life. As for the structure of the POLS, few studies investigated whether urban and rural populations differ in the covariation patterns of traits. A recent study on bird species showed that urbanisation may decouple the POLS covariation structure: slow-lived species are more risk-averse than fast-lived bird species, yet this POLS structure disappears in urban environments, mainly because slow-lived species seem to adjust their boldness based on how they perceive risk (Sol et al. 2018).

Although it was not my aim to study trait integration in response to urbanisation, several findings are worth discussing within the POLS framework. Urban damselflies had a shorter lifespan (chapter 5) and increased flight performance (chapter 6), suggesting a faster pace of life, yet in contrast they showed consistently slower larval growth rates across a range of temperatures (chapter 1). Further, urbanisation did not influence the behavioural traits, activity and boldness. Increased boldness in urban populations, for example, is often interpreted as a response to increased exposure to humans in urban areas (Miranda et al. 2013; Sol et al. 2013; Samia et al. 2015), although the same reasoning can hardly be applied for insects (Schuett et al. 2018) - especially so for aquatic insects, given the lack of human presence in aquatic environments. As for the covariation structure between traits, I found that activity and boldness behaviours were strongly correlated in urban, but not in rural damselflies; potentially an adaptative response to the more stressful urban environment. Together, this indicates that in the studied damselfly species, urbanisation affects many POLS traits, yet the urban-rural differentiation is not matching the POLS framework.

These results suggest that studying the POLS in response to urbanisation should include careful identification of urban stressors and corresponding response variables relevant for the study species. For example, immune responses, a relevant trait for urban-studies (as discussed below) also seem to be integrating in the POLS (Niemelä et al. 2012). Finally, recent work reveals that the pace-of-life syndrome can be sex specific (Debecker et al. 2016; Arnqvist et al. 2018; Hämäläinen et al. 2018; Lehmann et al. 2018), yet no work has been done to study this within the framework of urbanisation.

## Limitations and future directions

The three urban-related stressors on which I have focused in this thesis, namely UHI, pollution, and habitat fragmentation, certainly do not capture all characteristics of urban areas. One such stressor ignored in this study, artificial light in urban areas, is known to have a strong impact on animals living in the city (Gaston et al. 2015 and references therein), for example by altering the breeding phenology of birds (e.g. Kempenaers et al. 2010), or by effects on behaviour and fitness (Ouyang et al. 2018). Arthropods are affected as well (Davies et al. 2012; Knop et al. 2017), including aquatic insects (Perkin et al. 2011; Henn et al. 2014), whereby photoperiods are disrupted (Longcore and Rich 2004). Another factor, altered noise regimes in urban areas (Slabbekoorn and Ripmeester 2008) have long been recognized as a (novel) stressor, especially for vertebrates (Slabbekoorn and Peet 2003; Kight and Swaddle 2011). A recent study found that environmental noise also influences the predation rate of damselfly larvae (Villalobos-Jiménez et al. 2017). Finally, although I focused on higher mean temperatures associated with UHIs, differentiation in temperature variations between urban and rural areas is another aspect linked to the UHI effect (for the study region: Kaiser et al. 2016; Brans et al. 2018), which I have not considered in this thesis.

My primary goal was to test for adaptation to urban environments in damselflies by studying relevant response variables, yet several key traits remain unexplored in this context. Especially physiological traits that reflect the condition of an individual may be relevant endpoints to study in the context of urban stressors. Fatty acid profiles, for example, were found to be altered in urban-dwelling populations of birds, potentially due to dietary differences associated with urban areas (Andersson et al. 2015; Toledo et al. 2016; Isaksson et al. 2017). Oxidative damage (caused by an increase in reactive oxidants) has also been extensively studied in birds within the context of urbanisation, whereby factors associated with urban environments, such as pollution, artificial lights and altered diet quality, may drive increased oxidative stress (Hutton and McGraw 2015; Isaksson 2015; Isaksson et al. 2017; Herrera-Dueñas et al. 2017; but see Casasole et al. 2017). For the same reason, immune competence is also of interest for urban studies (Harris et al. 2013; Raap et al. 2017; Serieys et al. 2018).

While I tested life history across a range of temperatures, also flight performance is known to respond to ambient temperature experienced during the flight, and its TPC may potentially differ between urban and rural populations. Several studies with butterflies report increased flight performance (Merckx et al. 2006; Karlsson and Johansson 2008) or dispersal rate (Legrand et al. 2015; Kuussaari et al. 2016) with increasing temperatures. Hence, future studies should consider testing the flight performance of urban and rural populations at different ambient temperatures (as in e.g. Esterhuizen et al. 2014), which may provide additional insights into potential thermal adaptation to the higher temperatures encountered in cities. Testing for thermal adaptation in the adult stage is especially relevant given the stronger urban-rural differentiation in air compared to water temperatures.

One population factor that may differ between urban and rural populations and shape the expression of many traits is the population density. Changes in population density can result in altered density-dependent selection pressures on traits, hence influence trait expressions (Mueller 1997). Many studies reported altered abundance due to urbanisation (Fahrig 2003; McKinney 2008; but see e.g. Lowe et al. 2017), whereas the direction of change depends on the species in question (e.g. Shochat et al. 2010). Urbanisation-driven habitat fragmentation, for example, caused a strong decrease in population densities of the butterfly *Pieris rapae* (Rochat et al. 2017), whereas the UHI effects seems to be driving the increased abundance of the scale insect *Parthenolecanium quercifex* in urban areas (Meineke et al. 2013). Therefore, both negative and positive density-dependent selection may be driven by urbanisation. For example, higher winter survival rate in urban areas (due to human-provided food) can lead to increased competition for food during the breeding season, which in turn can result in lower clutch sizes and a decreased productivity in bird populations (Chamberlain et al. 2009).

Common garden rearing experiments, coupled with experimentally selected environmental factors (in our case urban-related stressors), are frequently applied to test for local adaptation, yet caution is warranted before drawing strong genetic inferences based on findings of these experiments (Kawecki and Ebert 2004; Kimberly and Salice 2015). Even though the role of phenotypic plasticity can be assumed to be limited, experiments where the study organism is reared for only one generation under common garden settings (as in this study) cannot fully disentangle maternal or epigenetic effects from genetic signals. Moreover, conditions in the laboratory may resemble either rural or urban environments, potentially confounding interpretation of adaptive responses. An even stronger approach to infer local adaptation would be reciprocal transplant experiments (Blanquart et al. 2013; Merilä & Hendry 2014), whereby individuals originating from rural and urban areas are translocated to both rural and urban locations, and their performance is compared at their native and foreign sites (Donihue & Lambert 2015). By further making use of molecular and genomic tools, testing of urbanisation-driven divergent selection, as well as revealing molecular mechanisms underlying phenotypic responses to urbanisation becomes possible (Schell 2018; see also Harris et al. 2013, 2015; Harris and Munshi-South; Perrier et al. 2017; Watson et al. 2017; Theodorou et al. 2018). Lastly, another shortcoming of the present study is the lack of intermediate (e.g. suburban) populations, which could have shed light on whether the urbanisation gradient drives gradual phenotypic change (as was shown in Merckx et al. in press).

One outstanding question is to what extent we can generalise the here reported responses to urbanisation. Meta-analyses suggest adaptations to urbanisation and urban stressors to be more or less consistent across locations (Chamberlain et al. 2009; Martinson and Raupp 2013; Alberti et al. 2017a; Sepp et al. 2018;). Yet, recent studies reveal a regional (e.g. latitudinal) aspect of responses to urbanisation (Diamond et al. 2015; Chown and Duffy 2015; Youngstead et al. 2017; Beasley et al. 2018), partly because the urban-rural differentiation in temperature has a regional signal (Chown and Duffy 2015). Given the strong latitudinal gradients in damselfly life history (e.g. Debecker et al 2017), it would be interesting to repeat the here studied urban-rural trait differentiation at different latitudes. This would also allow testing whether the gradual thermal evolution along latitudes is mirrored in the rapid thermal evolution along urbanisation gradients. This is also relevant to develop the idea that given the high temperature differences between urban and rural populations, urban populations can be used to predict what will happen in rural populations under future warming (so-called space-for-time approach; Dunne et al. 2004). It will be important to carefully consider for which traits this may hold. Indeed, we have seen that urban populations can also to some extent be considered as edge populations at moving range fronts, where spatial selection is at work, thereby precluding a space-for-time substitution for dispersal-related traits (unless for the assumedly common scenario where warming is associated with increased habitat fragmentation; see below).

As suggested above, the study of POLS evolution in response to urbanisation might benefit from comparisons with evolutionary changes during range expansions. This is because the newly colonized edge habitats are often more isolated and fragmented than the core habitats (Hill et al. 2011) and only the best dispersers arrive the range front; similar to urban areas. Matching my findings for urban populations, the flight performance and the underlying flight-related traits in the closely related damselfly *Coenagrion scitulum* that shows northwards range expansion were higher in edge populations (Swaegers et al. 2015; Therry et al. 2015), possibly due to ongoing spatial sorting. Yet, larvae from edge populations had a faster life history, i.e. faster growth and development rates and higher activity levels (Therry et al. 2014a, 2014c), which is suggested to be a response to lower densities (stronger r-selection) and lower temperatures (driving time constraints) at the range front. This contrasts with the observed evolution of slower growth rates in the warmer urban populations. Finally, it is worth testing whether the predicted pattern of more bold personalities at newly colonized/invaded habitats (Cote et al. 2010; Canestrelli et al. 2016) is also present in the here studied urban damselflies. I could not detect urban larvae being bolder, yet maybe this boldness pattern is only detectable in the adult stage.

Another perspective that future research on urbanisation should take into account is the temporal aspect of urbanisation. Responses to environmental change driven by urbanisation may include transient dynamics (Ramalho & Hobbs 2012). For example, the higher flight performance of urban damselflies may be a temporary phenomenon, analogous to the transient changes seen during range expansions, where ongoing range expansion creates constantly new fronts, and the high locomotor ability in the previously “edge” populations can be reversed (Chuang & Peterson 2016). Yet, range expansions differ in this context from an ongoing urbanisation process. Assuming that urban areas will be fragmented and isolated also in the future (unlike the transient status of “edge” habitats in range expansions), I expect a (more or less) stable scenario of a higher flight performance of urban damselfly populations, maintained by recurrent spatial sorting. The finding that sexual selection has the potential to further reinforce higher flight performance in urban populations of the study species corroborates this argument. Yet, the temporal aspect of urbanisation-driven trait differentiation remains to be tested, ideally using long-term monitoring of traits.

Finally, the here detected signals of rapid evolution may drive changes in ecological interactions, hence result in eco-evolutionary dynamics (Hendry 2016). So far this has been poorly studied in the context of urbanisation (Alberti 2015; Alberti et al. 2017b; Hendry et al. 2017; Schell 2018), but may be an important avenue for further research to better understand how aquatic ecosystems differ in urban and rural settings.

## Conclusions

In this thesis, I documented distinct responses to urban-related stressors between urban and rural populations of a damselfly, suggesting an evolutionary response to urbanisation. The higher urban temperatures seem to indirectly affect the life history of urban populations via altered growing season length, whereas larval behaviour was not altered by the UHI effect. When exposed to pesticides, urban damselfly larvae showed different behavioural responses when compared to rural larvae; both in terms of average behavioural expression and behavioural correlations. Finally, the more fragmentated habitats in urban areas seem to have selected for better flyers. Importantly, this differentiation seem to be driven by altered dispersal ecology, rather than traditional local adaptation. Taken together, in the studied damselfly I found clear signals of (assumedly) genetic differentiation between urban and rural populations that suggest differential selection imposed by three urbanisation-related stressors: pollution, heat and habitat isolation.

Notably, these stressors were acting in different life stages, highlighting the need for a full life cycle approach when studying the effects of urbanisation: spatial sorting due to increased fragmentation drives increased flight performance in urban adults, whereas local adaptation to increased pollution and temperature causes adaptive response in urban larvae. Importantly, these two mechanisms may be reinforcing or opposing each other (Figure 1 in the General introduction). Adults with good dispersal capacities will reach urban ponds, and potentially reproduce in this novel environment. Flight performance is probably to some extent heritable (in a closely related damselfly; Swaegers et al. 2015). Moreover, given that dispersal-associated traits such as locomotor performance, life-history and behavioural traits often covary (forming a so-called “dispersal syndrome”, Stevens et al. 2014), individuals newly arrived at urban ponds will be not only good flyers, but potentially also have distinct phenotypes in the larval stage when compared to rural individuals. In this scenario, the phenotype of the offspring of the new arrivals can be indirectly shaped by spatial sorting. This scenario was likely less important in our study system where local adaptation (to increased pollution and temperature) seemed the main driver of phenotypic differentiation observed in the larval stage. Furthermore, urban stress-induced negative effects on traits may carry over to the adult life stage. Another scenario can be suggested based on trade-offs; for example the slower growth rate of urban larvae may facilitate a better flight performance, which in turn might have been traded off with lifespan. Overall, findings of this study suggest that urbanisation may influence organisms via a complex network of intertwined ecological and evolutionary dynamics such as trade-offs, syndromes, ontogenic (de)coupling, and stage-specific selection pressures.

As cities are warmer than the surrounding rural areas, studying populations that inhabit urban areas can offer unique insight into the expected thermal responses of organisms in the face of climate change (as suggested by e.g. Farrell et al. 2015; Chown and Duffy 2015). Other prominent urbanisation-related stressors tested in this project are also expected to increase in intensity in the near future: the rate of pesticide application has been steadily increasing (Bernhardt et al. 2017), and habitat fragmentation is expected to accompany the ongoing urbanisation process (Parris 2016). Therefore, I further propose the use of urban areas as experimental settings for the study of adaptation to contaminant exposure and habitat fragmentation, and in general to predict species responses to anthropogenic activities.

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## SUMMARY

The rate of urbanisation is increasing on a global scale. Compared to rural areas, cities are warmer, more polluted by chemicals, and more fragmented. Although biodiversity is clearly impacted by this anthropogenic activity, there are large gaps in our understanding on the effects of urbanisation at the population and individual levels. In this thesis, I tested whether urban and rural populations of the damselfly *Coenagrion puella* differed in their responses to higher temperatures, increased contamination, and more intense habitat fragmentation. For this, I mostly applied a common garden experimental approach, either in the laboratory or in semi-natural outdoor conditions, allowing the identification of genetic adaptation. To also test the outstanding question whether urbanisation influences sexual selection regimes, I additionally did an experiment with field-collected adult damselflies. All study populations were situated in Flanders, Belgium.

I found strong divergence between urban and rural populations in responses to each of the three studied urbanisation-related stressors, suggesting evolutionary responses to urbanisation. The higher temperatures in urban areas influenced the life history of urban damselflies by altering growing season length, yet did not affect behavioural traits. With regard to contamination, damselfly larvae from urban habitats showed different behavioural responses to pesticides when compared to rural larvae. Furthermore, pesticide exposure differently affected behavioural correlations in urban and in rural larvae. Interestingly, several responses to urban-related stressors strongly depended on the sex of the damselfly, as well as the life stage at which they were exposed to the stressor. Finally, the more fragmentated habitats in urban areas seem to have selected for better flyers by influencing the dispersal ecology of damselflies, which was further strengthened by sexual selection for a higher flight performance in urban populations.

In conclusion, I found clear effects of urbanisation on the evolution and ecology of a damselfly. My findings suggest that differential selection was imposed by the here tested urbanisation-related stressors. Importantly, these stressors were acting in different life stages, highlighting the need for a full life cycle approach when studying the effects of urbanisation.

# SAMENVATTING

Verstedelijking neemt wereldwijd toe. In vergelijking met rurale gebieden zijn steden warmer en gekenmerkt door een hogere verontreinigingsgraad en habitat fragmentatie. Hoewel biodiversiteit duidelijk beïnvloed wordt door deze vormen van antropogene activiteit en verstoring, is het minder duidelijk wat de gevolgen van verstedelijking zijn op het niveau van individuen en populaties. In deze thesis testte ik of urbane en rurale populaties van de waterjuffersoort *Coenagrion puella* verschillen in hun responsen op toenemende temperatuur, verhoogde contaminatie, en intensere habitat fragmentatie. Mijn experimentele methoden bestaan voornamelijk uit het uitvoeren van 'common garden' experimenten, zowel in het labo als in semi-natuurlijke omgevingscondities (veld experimenten), wat ons in staat stelt genetische adaptatie te onderzoeken. Daarnaast voerde ik een experiment uit met waterjuffers rechtstreeks afkomstig uit hun natuurlijke populaties, en testte ik of en hoe verstedelijking seksuele selectie regimes beïnvloedt.

De resultaten van mijn common garden studies tonen een sterke differentiatie tussen urbane en rurale populaties in respons op de drie verstedelijkings-gerelateerde stressoren (temperatuur, pollutie, fragmentatie), wat een aanwijzing is voor urbane evolutie. Hogere temperaturen in de stad beïnvloeden de levensgeschiedeniskenmerken van waterjuffers uit de stadsomgeving, vermoedelijk via een verlengd groeiseizoen; gedragskenmerken daarentegen toonden geen duidelijke aanwijzing voor gedragsadaptatie in urbane populaties als respons op verhoogde temperaturen. Met betrekking tot pollutie konden we aantonen dat, in vergelijking met rurale waterjuffers, waterjuffers uit de stad gekenmerkt zijn door een gewijzigd gedrag (activiteit) als respons op blootstelling aan pesticiden. Bovendien vertoonden urbane en rurale populaties een verschillend covariatiepatroon tussen gedragskenmerken bij blootstelling aan pesticiden. Verschillende kenmerkresponsen bij blootstelling aan de verschillende verstedelijkings-gerelateerde stressoren waren afhankelijk van het geslacht van de waterjuffers, alsook het levensstadium waarin het individu zich bevond bij de blootstelling aan de specifieke stressor. Tot slot vonden we dat urbane gebieden, gekenmerkt door een hogere mate van habitatfragmentatie, vermoedelijk via de impact op de dispersie van de waterjuffers, selectie voor een hogere vliegcapaciteit

veroorzaken. Bovendien bleek een versterkend effect van seksuele selectie voor een hogere vliegcapaciteit aanwezig in de urbane populaties.

Samengevat tonen de resultaten van mijn onderzoek aan dat verstedelijking een duidelijk effect heeft op evolutionaire en ecologische processen in waterjufferpopulaties in Vlaanderen. Mijn onderzoek wijst op een verschillende selectiedruk door de onderzochte selectiefactoren in de stad in vergelijking met het platteland. Deze stressoren beïnvloedden de onderzochte waterjufferpopulaties bovendien verschillend afhankelijk van het levensstadium van de individuen. Dit wijst duidelijk op de noodzaak voor onderzoek op verschillende levensstadia geïntegreerd in de volledige levenscyclus bij onderzoek naar de effecten van verstedelijking op organismen.

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