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**Cryptic eco-evolutionary feedback in the city: urban evolution of prey dampens the effect of urban evolution of the predator**

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## ABSTRACT

1. Most research on eco-evolutionary feedbacks focuses on ecological consequences of evolution in a single species. This ignores the fact that evolution in response to a shared environmental factor in multiple species involved in interactions could alter the net cumulative effect of evolution on ecology.
2. We empirically tested whether urbanization-driven evolution in a predator (nymphs of the damselfly *Ischnura elegans*) and its prey (the water flea *Daphnia magna*) jointly shape the outcome of predation under simulated heatwaves. Both interactors show genetic trait adaptation to urbanization, particularly to higher temperatures.
3. We cross-exposed common-garden reared damselflies and *Daphnia* from replicated urban and rural populations, and quantified predation rates and functional response traits.
4. Urban damselfly nymphs showed higher encounter and predation rates than rural damselflies when exposed to rural prey, but this difference disappeared when they preyed on urban *Daphnia*. This represents a case of a cryptic evo-to-eco feedback, where the evolution of one species dampens the effects of the evolution of another species on their interaction strength. The effects of evolution of each single species were strong: the scenario in which only the predator or prey was adapted to urbanization resulted in a ca. 250% increase in encounter rate and a ca. 25% increase in predation rate, compared to the rural predator - rural prey combination.
5. Our results provide unique evidence for eco-evolutionary feedbacks in cities, and underscore the importance of a multi-species approach in eco-evolutionary dynamics research.

**Key words:** cryptic urban eco-evolutionary feedbacks, *Daphnia*, heatwaves, *Ischnura*, predator-prey interactions, thermal adaptation.

## ABSTRACT (DUTCH)

1. Onderzoek naar eco-evolutionaire terugkoppelingen focust vaak op de ecologische gevolgen van evolutie in één soort. Bijgevolg negeert men de mogelijkheid dat evolutionaire veranderingen van meerdere interagerende soorten als respons op een gedeelde omgevingsverandering met elkaar kunnen interfereren en zo het netto effect van evolutie op ecologische processen kan veranderen.
2. We testten empirisch of door verstedelijking gedreven evolutie in een predator (larven van de waterjuffer *Ischnura elegans*) en zijn prooi (de watervlo *Daphnia magna*) de uitkomst van predatie onder gesimuleerde hittegolven beïnvloedt. Beide interactoren vertonen genetische adaptatie aan de stadsomgeving, meer specifiek de daar voorkomende hogere temperaturen.
3. We stelden waterjuffers en watervlooien, afkomstig van gerepliceerde stedelijke en rurale populaties en opgegroeid in een gestandaardiseerde laboratorium-omgeving, bloot aan elkaar volgens een experimenteel design waarbij alle combinaties van herkomst van prooi en predator werden getest. We kwantificeerden telkens de predatiesnelheid en functionele respons-kenmerken.
4. Stedelijke waterjufferlarven vertoonden een hogere ontmoetings- en predatiesnelheid dan larven afkomstig uit rurale gebieden wanneer ze blootgesteld werden aan rurale *Daphnia*, maar niet wanneer ze blootgesteld werden aan *Daphnia* afkomstig uit de stad. Deze bevinding wijst op een cryptische evo-naar-eco terugkoppeling, waarbij evolutie in één van de soorten het effect van evolutie van de andere soort op de sterkte van hun interactie dempt. De effecten van evolutie in elke soort apart waren sterk: de scenario's waarin enkel de predator of de prooi aangepast was aan de stadsomgeving resulteerde in een ca. 250% stijging van ontmoetingssnelheid en een ca. 25% verhoging van de predatiesnelheid, in vergelijking met een situatie waarbij een rurale predator met een rurale prooi was gecombineerd.
5. Deze resultaten leveren uniek bewijs voor een eco-evolutionaire terugkoppeling in steden, en onderlijnen het belang van een multi-soorten benadering in het onderzoek naar eco-evolutionaire dynamieken.

## INTRODUCTION

Evolution can occur at small temporal and spatial scales, and can lead to trait changes that can profoundly influence ecology, including population dynamics, interaction strengths, community composition, and ecosystem functioning (Pantel et al. 2015, Kinnison et al. 2015, Hendry 2016, De Meester et al. 2019). Such ‘broad sense’ eco-evolutionary feedbacks (Hendry 2017, De Meester et al. 2019) may fundamentally change species interaction dynamics, including predation (De Meester et al. 2019). Most eco-evolutionary dynamics research focuses on consequences of evolution in one species and in simplified settings, providing proof-of-principle rather than quantifying eco-evolutionary feedbacks across realistic landscapes (De Meester et al. 2019). Empirical (De Meester et al. 2019) and modelling (Vanoverbeke et al. 2016, Cortez 2018, Thompson and Fronhofer 2019) studies on eco-evolutionary feedbacks in which the evolution of multiple interacting species to the same stressor, hence not in the context of co-evolution, is taken into consideration are very rare,

When quantifying the ecological effect of evolution in a species of a community, the effect of non-equilibrium settings in which only one of the species was allowed to evolve is measured. This may reflect reality in cases where rates of evolutionary trait change differ among species (Hendry and Kinnison 1999) or in which interacting populations have a different evolutionary history (e.g. upon encounter after dispersing into a novel habitat). Such studies provide an estimate of the potential impact of evolution in a particular species on the interaction strength with other species. However, if different partners involved in species interactions evolve simultaneously to the same stressor, they might increase or decrease the net cumulative effect of evolution on their interaction strength. Eco-evolutionary dynamics research across realistic systems and landscapes urgently requires to test how eco-evolutionary feedbacks are impacted by the joint evolution of interacting species. Such dynamics, well-known in the context of co-evolution (where species directly evolve in response to selection imposed by each other), are less understood in the context of independent evolutionary responses in multiple species to environmental change, including human-induced selection pressures (Urban et al. 2016).

A key challenge in identifying and quantifying eco-evolutionary dynamics in nature is that the effects of evolution are often compensatory, counteracting environmentally-induced phenotypically plastic changes (Ellner et al. 2011, Kinnison et al. 2015). Evolution in response to an environmental change thus can result in trait differentiation across space that dampens the ecological changes that are expected to result from the environmental challenge, and consequently reduces the net ecological impact across the gradient (Ellner et al. 2011, Kinnison et al. 2015, Urban et al. 2020). This makes the detection of eco-evolutionary feedbacks less straightforward, as it may be the absence of any ecological change across environmental gradients that indicates eco-evolutionary feedbacks (Urban et al. 2020). In a multispecies context, this dampening effect of evolution can also extend to evolution in one species buffering the ecological effects of evolution in another species (Nadeau and Urban 2019). The combined effect of evolutionary trait change in multiple species will thus not necessarily result in an increasing net contribution of trait evolution to ecology. Evolution in one species may dampen the evo-to-eco effects that originate from the evolution in the other species. Indeed, the net effect of trait evolution in multiple species on ecology might be smaller than the effect of evolution in a single species, and may even be reduced to zero, thereby masking the ecological effect of the evolution in the single species. This would generate eco-evolutionary dynamics that are ‘cryptic’ (Kinnison et al. 2015).

Urbanization is associated with strong and novel abiotic and biotic selection pressures (Grimm et al. 2008, Pickett et al. 2016) and can lead to rapid non-adaptive (i.e. as a consequence of neutral genetic drift, gene flow, and chance mutations) and adaptive (i.e. in response to natural and sexual selection) evolution, as shown by the recent burst of studies covering plants, animals, and micro-organisms (Johnson and Munshi-South 2017, Szulkin et al. 2020). Several studies have reported adaptive genetic responses to urban warming (Diamond et al. 2017), pollution (Whitehead et al. 2017), and fragmentation (Cheptou et al. 2017). Given the steep environmental gradients and the observed patterns of microgeographic adaptation, cities are excellent model systems to quantify how evolutionary trait change affects ecological processes and to what extent these eco-evolutionary feedbacks (‘broad sense’, Hendry 2017, De Meester et al. 2019) meaningfully impact ecosystem

functions and services in cities (Alberti et al. 2017, Brans et al. 2020, Des Roches et al. 2020). So far, however, attempts to quantify these feedbacks in cities are limited (Brans et al. 2017a), and no study has explored whether urban evolution in multiple species leads to intensified, or potentially dampened evo-to-eco effects, and thus cryptic eco-evolutionary dynamics.

Predation is a key trophic interaction structuring pond communities, for which eco-evolutionary dynamics have been repeatedly shown (Hairston et al. 2005, Yoshida et al. 2007, Palkovacs and Post 2008, Bassar et al. 2010). It is well documented that genetic adaptation in prey to a specific predator can change predator-prey dynamics (Yoshida et al. 2007). It is, however, likely that adaptation of prey or predator to environmental change, like changes in temperatures, can similarly induce changes in interaction strengths of the predator-prey interaction (Tüzün and Stoks 2018). Moreover, adaptation of both predator and prey to the same environmental gradient can lead to additional interactions that may amplify or reduce the net effect of evolution on interaction strengths. In one example, De Block et al. (2013) indeed reported that the outcome of predator-prey interactions between nymphal damselfly predators (*Ischnura elegans*) and water flea prey (*Daphnia magna*) did not only depend on temperature during the predator trials, but also on the latitude of origin of both predator and prey (animals originated from three source latitudes spanning >1500 km).

We here tested the hypothesis that urban evolution of both predator and prey jointly drives the outcome of their short-term trophic interaction and quantified to what extent this interaction is compensatory, leading to a cryptic eco-evolutionary feedback. We thereby used a text-book example of a predator-prey interaction between nymphs of the damselfly *Ischnura elegans* and its prey the water flea *Daphnia magna* (Begon et al. 2005). We capitalized on the adaptation to urban heat islands in both damselflies and water fleas in the study area (Flanders, Belgium). The majority of these responses are linked to the thermal regimes typical for urban areas (Wouters et al. 2017, Brans et al. 2018b). Urban ponds in Flanders are warmer compared to rural ponds (up to 3°C and 4°C warmer in mean and maximum daily summer temperatures, respectively) and have longer estimated growing seasons (Brans et al. 2018b). Moreover, urban areas in Flanders experience more intense and longer heat periods (Lauwaet et al. 2018), which is predicted to be further exacerbated with future climate

change (Wouters et al. 2017). Urban evolution of a slower growth rate (in response to relaxed time constraints for nymphal development during the longer growing seasons in urban areas), and a higher ability to cope with heat stress in terms of energy availability (in response to the stronger and more frequent heat waves in urban areas) have been shown in *Coenagrion* damselfly nymphs (Tüzün et al. 2017, Tüzün and Stoks 2021), a genus closely related to *Ischnura*. Urban evolution of a higher heat tolerance, haemoglobin content, faster pace-of-life, higher level of energy storage molecules and an altered stress physiology has been documented in the water flea *D. magna* (Brans et al. 2017b, 2018a, Brans and De Meester 2018).

Using predators and prey sampled from replicated urban and rural ponds in the study area, we first assessed multiple fitness-related traits in both *Ischnura* (development time, heat tolerance, body mass) and *Daphnia* (body size) in common-garden settings to confirmed previously reported genetic differentiation between urban and rural populations of both damselflies and water fleas. We then quantified predation rates of urban and rural damselfly predators, reared under different rural and urban thermal regimes and being exposed to a five-day heatwave, when cross-exposed to urban and rural water flea prey, that had undergone the same thermal rearing and heatwave conditions. To get mechanistic insights in the predation rates, we thereby also directly estimated encounter rate (the inverse of the time between successive encounters) and handling time, two important determinants of the functional response of a predator (Jeschke et al. 2002, Begon et al. 2005). The effects of extreme temperature events, which can be lethal and are fundamentally different from increases in average temperatures as organisms are challenged at the extremities of their thermal tolerance range (Sinclair et al. 2012, Sentis et al. 2013a, 2017), may thus trigger evo-to-eco feedbacks shaped by thermal adaptation. Predation rates often increase with increasing average temperatures, yet decrease rapidly at extreme temperatures as a result of decreased searching activities and encounter rates, in combination with longer handling times (Sentis et al. 2013b, Stoks et al. 2017). We predicted that thermal adaptation to higher temperatures in urban damselfly populations might allow them to sustain higher predation activities during a heatwave compared to rural predators. Likewise, evolution of a higher heat tolerance in urban water flea prey might reduce their sensitivity to thermal stress, lowering



their vulnerability to predation. We additionally hypothesized that scenarios in which both the predator and prey evolve to urban habitats lead to predatory-prey interactions that differ from scenarios in which either only the prey or the predator evolve. More specifically, we predicted that the net effect of evolution on predator-prey interaction strength might be reduced if both predator and prey evolve, leading to a cryptic evo-to-eco feedback (i.e. urban evolution to predator-prey interactions).

## METHODS

### Collecting and rearing of *Ischnura elegans* predator and *Daphnia magna* prey

Both damselfly (*Ischnura elegans*) predators and water flea (*Daphnia magna*) prey originated from replicated urban and rural ponds in Flanders (Belgium; details in Table S1 and Fig. S1, Appendix - section A. Methods). To avoid interference from local adaptation to a given pond, as well as the potential of co-evolution of the predator and prey study populations, we used different sets of urban and rural ponds for predator and prey. Flanders is densely populated (ca 700 inhabitants/ km<sup>2</sup>, IBZ, 2021). We used percentage built-up area (BA) in the regional surroundings of the pond [based on the Large-scale Reference Database, LRD (2013)] to assess urbanization levels (urban/rural) of study populations (as in Brans & De Meester, 2018; Brans et al. 2017a,b, 2018, detailed in Appendix - section A. Methods). %BU, a metric including housing and building infrastructure but excluding roads and parking lots, has shown to be a reliable proxy of urbanization as it, in the specific study region, positively correlates with impervious substrate cover (e.g. roads) and artificial constructions (e.g. bridges), and is negatively associated with the area of seminatural habitats (Piano et al., 2020). In addition, rural locations had to meet the criterion of a minimum 20% biologically valuable area (detailed in Appendix - section A. Methods) to prevent sampling populations in non-urban agricultural areas.

Rearing conditions for both *Ischnura* and *Daphnia* during the pre-experimental generations are detailed in Appendix (section A. Methods). In short, nymphs hatched from eggs obtained of field-mated *Ischnura* females from three urban and three rural populations, were individually reared at

standardized laboratory conditions (ad libitum daily feeding with *Artemia* nauplii, 14:10 L:D photoperiod). Nymphs were reared at either 20 or 24°C ( $\pm 0.5^\circ\text{C}$ ) using temperature-controlled water baths, until reaching the final nymph stage, and then assigned to a specific heatwave treatment (see further). For *Daphnia*, three clones from each of four urban and four rural populations (total  $n=24$  clones) were kept in the laboratory for several generations; clones were first cultured for two generations (20°C; 16:8 L:D photoperiod; 100000 cells/mL of the green algae *Acutodesmus obliquus*) to obviate interference from (grand)maternal effects, after which for each clone, triplicate cohorts (12 individuals in 500 mL dechlorinated tap water) were reared for two experimental generations at 20 and 24°C ( $\pm 0.9^\circ\text{C}$ ) in a temperature-controlled room under standardized conditions (100000 cells/mL *Acutodesmus obliquus*, 14:10 L:D photoperiod, two-daily water refreshment of 80%). New generations were started up using 2<sup>nd</sup> to 4<sup>th</sup> clutch offspring, which enabled to standardize the number of generations and time in the lab across all populations. From these cohorts, newborn juveniles were transferred to the heatwave treatments

*Ischnura* nymphs were reared in the common-garden setting for one generation, from egg to final instar. While we cannot exclude maternal effects to partly shape observed differences among urban and rural predator nymphs, Shama et al. (2011) showed maternal effects to play a minor role in shaping life-histories of the same species. Differences in life history traits observed in our experiment can thus be confidently interpreted as largely reflecting genetic differences. *Daphnia* prey genotypes were reared for multiple generations under common-garden conditions, ensuring that (grand)maternal effects do not interfere with genetic differences in determining trait values. *D. magna* reproduces by cyclical parthenogenesis, so that genotypes can be kept as clonal lineages in the laboratory during many generations.

## **Experimental design and procedures**

### *General set-up*

Both predator and prey were separately reared at 20 or 24°C (mimicking mean rural and urban summer temperatures for ponds in the region, Brans et al. 2018b, Wouters et al. 2017) and exposed to a 4°C or 8°C temperature increase to reach heatwave temperatures of 28°C (rural areas) and 32°C

(urban areas) resulting in three heatwave treatments (Fig. 1): HW20-28°C (20°C rearing temperature and 28°C heatwave temperature), HW24-28°C (24°C rearing temperature and 28°C heatwave temperature), and HW24-32°C (24°C rearing temperature and 32°C heatwave temperature). As we focused on testing the effects of urbanization background and thermal acclimation on predation rates under thermally challenging conditions, all animals were exposed to a simulated heatwave. Moreover, as testing our aims did not require a full factorial design, we did not install two thermal treatments: individuals reared at 20°C did not undergo a 4°C temperature increase to 24°C, as this does not result in a thermally challenging condition. A temperature increase from 20°C to 32°C was also not implemented, as this temperature difference is less commonly observed in the study region. Predation trials were video-recorded (Sony HDR-CX 240, recorded at 25 fps) to later extract two functional response parameters (encounter rate and handling time).

The full experimental set-up [2 urbanization levels (rural/urban origin)  $\times$  2 species (predator/prey)  $\times$  3 heatwave treatments (HW20-28°C/HW24-28°C/HW24-32°C)] resulted in a total of 12 combinations, which were replicated 12 times (total of 144 predation trials) (Fig. 1). Independence of replicates was maximized via a randomization across origin of clonal lineages and *Ischnura* mother identity (i.e. ‘family’) (see Appendix – section A. Methods).

#### *Heatwave treatment and predation trials*

Both damselfly nymphs and water flea cohorts (30 newborn individuals, fed *ad libitum* throughout the heatwave with the green algae *Acutodesmus obliquus*, eliminating possible intraspecific competition) passed separately through a simulated five-day heatwave of either 28 or 32°C (HW20-28°C/HW24-28°C/HW24-32°C). At the end of the heatwave period, when any effects of the heatwave on predator and prey are assumed to be most pronounced, 1h-predation trials took place at the associated heatwave temperature. This set-up enabled us to measure functional response parameters (encounter rate, handling time, and predation rate) in a standardized and controlled way without interference of numerical effects.

The procedures related to the heatwave exposures are detailed in Appendix (section A. Methods). In short, the simulated 5-day heatwaves consisted of a gradual increase in temperature over the first two days, followed by two days at the peak temperature (detailed in Appendix- section A. Methods). The medium for the *Daphnia* was renewed on the first and third day of the heatwave period (with medium incubated at the appropriate water temperature of the specific heatwave condition). At the end of the heatwave (day five), 20 randomly chosen *Daphnia* of the cohort were inoculated into a 2l aquarium (180×133×126 mm) with fresh medium (at the associated heatwave temperature), and allowed one hour of acclimatization. Another three randomly chosen *Daphnia* per cohort were measured for body size (Brans et al. 2017a, see Appendix - section A. Methods). Thereafter, one damselfly nymph (starved for 24h before the predation trial) of the same heatwave treatment, and according to experimental combinations represented in Figure 1, was added to the trial aquarium, and a 60-minute predation trial started. In some cases (n=6 out of 144) *Daphnia* mortality after the heatwave in a clonal cohort was present, hence <20 (range: 16-19) *Daphnia* could enter the predation trial. After each predation trial, damselflies were scored for CT<sub>MAX</sub> (critical thermal maximum, i.e. the temperature at which the animal fails to maintain motoric functioning and faints), using the same procedure as in Op de Beeck et al. (2017), weighted, and sexed (see Appendix - section A. Methods for details).

## **Response variables**

Measurement of the variables to characterize adaptation to the urban environment (heat tolerance, body mass, and development time for predator, and body size for prey) were conducted based on standardized measurement protocols (for specifics see Appendix - section A. Methods).

Predation rates were expressed as  $N_{\text{eaten}}/N_{\text{initial}}$  of *Daphnia* after one hour in the trial. Using the video recordings of each trial, we directly calculated two functional response parameters, encounter rate and handling time based on the three first successful attacks (Jeschke et al. 2002, Novak and Wootton 2008, Okuyama 2010, Twardochleb et al. 2020). The encounter rate ( $s^{-1}$ ) was calculated as the inverse of the searching time, which is the length of the time interval from the moment the predator finished consuming a prey item to the moment a next successful attack took place. Note that

for predators with negligible attack times, e.g. for sit-and-wait predators such as damselfly nymphs, calculating encounter rates using only successful attacks is expected to result in reliable encounter rate estimates (Jeschke et al. 2002). In our experiment, encounter rate was quantified twice per trial: searching time for the second and third prey item. Note that no searching time was calculated for the first captured prey, given the time needed to find the first prey is confounded with the time needed for the predator to familiarize with the new environment. The handling time, defined as the duration that a predator spends on consuming a captured prey was calculated as the length of the time interval from the moment of capturing the prey to the moment of finishing prey consumption (three estimates per trial, handling time of the first three prey items).

### Statistical analyses

All statistical analyses were conducted with R v3.5.3 (3.5.1. 2018), using the packages ‘lme4’ for linear models (Bates et al. 2015), ‘lmerTest’ for F-test of fixed effects (error calculation Type III sums of squares) (Kuznetsova et al. 2017) and ‘emmeans’ for Tukey’s post-hoc pairwise comparisons following significant interaction effects (Lenth 2020). To meet model assumptions of normality, encounter rate and handling time were log transformed. All models conformed with assumptions of homogeneity of variances and regression slopes.

Statistical analyses on *Ischnura* body mass, development time and  $CT_{MAX}$ , and *Daphnia* body size are detailed in Appendix (section A. Methods).

### *Encounter rate and handling time*

We computed a linear mixed-effect model to test for an effect of urbanization background of both predator and prey, the heatwave treatment, and their interactions, on the two measured functional response parameters of the predator (see simplified model formula). Additionally, predator sex and time point of attack were included as fixed effects, and predator body mass and prey size as covariates. As random effects we included the population of origin of predator and prey (nested in urbanization background), the family (for *Ischnura*) and clone (for *Daphnia*) nested in population, and the date of the start of the heatwave (see below for the model syntax in R language). Given encounter

rates and handling times were assessed based on the first three successful attacks, a repeated measures structure for each estimate (two for encounter rate, three for handling time) was included by including trial ID as an additional random effect.. While both variables can be impacted by prey size and predator body mass, (Thompson 1978), adding or removing *Daphnia* size and *Ischnura* body mass to the models did not qualitatively change the results (see Results and Appendix - section B. Results). We nevertheless kept prey size and predator mass (as covariates) in the final models. This resulted in following model: response ~ *Ischnura* urbanization background × *Daphnia* urbanization background × heatwave treatment + *Ischnura* sex + *Ischnura* mass + *Daphnia* size + time point + (1 | *Ischnura* population / *Ischnura* mother) + (1 | *Daphnia* population / *Daphnia* clone) + (1 | trial date) + (1 | trial ID).

#### *Predation rates*

We computed a similar linear mixed-effect model as for the functional response parameters to test for an effect of urbanization of predator and prey, and heatwave treatments on predation rates (fixed effects: urbanization background of both predator and prey, and the heatwave treatment) (see simplified model formula). We included the specific wet mass of *Daphnia* and damselfly (corrected for the number and specific mass of eaten *Daphnia* as before) as covariates for each trial, and *Ischnura* sex as fixed effect in the analyses. As random effects we included the population of origin of predator and prey (nested in urbanization background), the family (for *Ischnura*) and clone (for *Daphnia*), nested in population, and the date of the start of the heatwave. For the same reason as mentioned for handling time and encounter rate, we included predator mass and prey size as covariates. The model without mass and size did not qualitatively change the results; see Results and Appendix - section B. Results (but see below for an additional model upon the detection of a significant effect of prey size on predation rates). Testing models using *Daphnia* body mass instead of size did not qualitatively change the results (see Results and Appendix - section B. Results). This resulted in following model: predation rate ~ *Ischnura* urbanization background × *Daphnia* urbanization background × heatwave treatment + *Ischnura* sex + *Ischnura* mass + *Daphnia* size + (1 | *Ischnura* population / *Ischnura* mother) + (1 | *Daphnia* population / *Daphnia* clone) + (1 | trial date).

Finally, to account for potential effects of geography beyond rural and urban designations on the tested response variables (encounter rate, handling time, predation rate), we constructed separate models with the same structures as described above, that additionally included latitude and longitude of both predator and prey populations as covariates. As these models resulted in qualitatively similar effects of urbanization levels of the prey and predator (and their interaction), we exclude these covariates from the final models, but report the full statistical results in the Appendix (section B. Results)

## RESULTS

Results on *Daphnia* body size and *Ischnura* development time, heat tolerance ( $CT_{MAX}$ , Critical Thermal Maximum), and body mass are detailed in Appendix (section B1. Results, Figures S2- S5, Tables S2 and S3), and confirm previously reported differences between urban and rural populations of predator and prey species. In short, urban *Ischnura* predator nymphs had a ca. 15 day longer development time ( $p=0.001$ ) (see Tüzün et al. 2017) and ca. 5% higher body mass ( $p=0.008$ ) compared to rural ones, whereas they did not differ in  $CT_{MAX}$  ( $p=0.834$ ). Urban *Daphnia* prey were ca. 5% smaller ( $p=0.036$ ) compared to rural *Daphnia* (see Brans et al. 2017b, Brans and De Meester 2018).

### Encounter rate and handling time

Encounter rates ( $s^{-1}$ , the inverse of time used for searching prey), were significantly impacted by the interaction between urbanization backgrounds of predator and prey ( $F_{1/1231.6}=16.111$ ,  $p<0.001$ , Table 1A, Fig. 2A,B). Urban *Ischnura* had a higher encounter rate compared to rural *Ischnura* in the presence of rural *Daphnia* (post-hoc test,  $p=0.007$ , Fig. 2B, Table S4, Appendix - section B2. Results), but not in the presence of urban *Daphnia* ( $p=0.104$ , Fig. 2B, Table S4). Rural *Daphnia* were encountered faster than urban *Daphnia* by urban *Ischnura* ( $p=0.023$ , Fig. 2B, Table S4), while the opposite pattern was observed in the presence of rural *Ischnura* ( $p=0.025$ , Fig. 2B, Table S4). Results (Fig. 2A) suggests that this interaction effect is most strongly developed in the heatwave treatment mimicking urban conditions (HW24-32°C, Table 1A, see also Table S4).

Handling times (s, the duration a predator spends on prey consumption) were jointly driven by both the urbanization backgrounds of the prey and predator, and the heatwave treatment (cf. Heatwave  $\times$  Urbanization predator  $\times$  Urbanization prey,  $F_{2/146.3}=5.090$ ,  $p=0.007$ , Table 1B, Fig. 2D,E). In the treatment mimicking urban conditions with a strong heatwave (HW24-32°C), urban *Ischnura* had a shorter handling time compared to rural *Ischnura* in the presence of rural *Daphnia* (post-hoc test,  $p=0.033$ , Table S5, Appendix - section B2. Results), but had a similar handling time in the presence of urban *Daphnia* ( $p=0.502$ , Table S5). In this heatwave, rural *Daphnia* were handled faster than urban *Daphnia* in the presence of urban *Ischnura* ( $p=0.006$ , Table S5), whereas handling times were similar for rural and urban *Daphnia* eaten by rural *Ischnura* ( $p=0.855$ , Table S5).

Encounter rates decreased ( $F_{1/144.49}=5.552$ ,  $p=0.020$ , Table 1A) and handling times ( $F_{2/294.6}=7.002$ ,  $p=0.001$ , Table 1B) increased with successive attacks.

### Predation rate

Predation rates ( $N_{\text{eaten}}/N_{\text{initial}} \cdot h^{-1}$ , ratio of eaten prey to initial prey) were significantly impacted by the interaction between the urbanization backgrounds of predator and prey ( $F_{1/121.895}=5.912$ ,  $p=0.016$ , Table 1C, Fig. 2G,H). Urban *Ischnura* had a higher predation rate compared to rural *Ischnura* in the presence of rural *Daphnia* (post-hoc test,  $p=0.021$ , Fig. 2H, Table S6, Appendix - section B2. Results), but both had a similar predation rate in the presence of urban *Daphnia* ( $p=0.680$ , Fig. 2H, Table S6). In reverse, rural *Daphnia* suffered higher predation than urban *Daphnia* in the presence of urban *Ischnura* ( $p=0.034$ , Fig. 2H, Table S6), but not in the presence of rural *Ischnura* ( $p=0.718$ , Fig. 2H, Table S6). The heatwave treatment affected the predation rates ( $F_{2/107.604}=6.687$ ,  $p=0.002$ , Table 1, Fig. 2G): predation rates were higher at HW20-28°C than at HW24-28°C (post-hoc test,  $p=0.019$ , Table S6) and HW24-32°C ( $p=0.008$ , Table S6), but did not differ between the latter two heatwaves ( $p=0.984$ , Table S6). Predation rates increased with smaller prey size ( $\beta=-0.048 \pm 0.014$ ,  $p=0.003$ , Table 1C).

### DISCUSSION



By applying a two-species common-garden approach, we demonstrated how independent evolution in response to urbanization in both damselfly predators and water flea prey affects their trophic interaction under thermal stress. Notably, our results provide a unique example of a cryptic evo-to-eco feedback ('broad sense' *sensu* De Meester et al. 2019, Hendry 2017) in the context of urbanization, in which urban evolution of predator and prey only leads to differences in encounter and predation rates when the urbanization backgrounds of predator and prey do not match. In the next paragraphs we detail our findings and elaborate on potential mechanisms underlying them.

*A cryptic evo-to-eco feedback: urban evolution in the prey dampens the ecological effect of urban evolution in the predator in a thermally challenging environment*

While eco-evolutionary feedbacks in which evolution of one species to a variable extent dampens the effects of evolution in another species are well-documented in cases of co-evolution (geographic mosaic of co-evolution, Thompson 2005; host-parasite co-evolution, Decaestecker et al. 2007; sympatric predator-prey interactions, Hairston et al. 2005), our results provide a rare demonstration of these feedbacks in response to independent evolution of two species as they adapt to a recurrent, common geographic gradient. This may lead to 'cryptic eco-evolutionary dynamics' (Kinnison et al. 2015), whereby the ecological effects of evolution are only revealed in common-garden experiments where predators and prey from different backgrounds are confronted with each other. More specifically, encounter rates of urban damselflies were higher compared to rural damselflies when encountering rural *Daphnia*, but similar when feeding on urban *Daphnia*. Conversely, encounter rates with urban *Daphnia* were lower compared to those with rural *Daphnia* in confrontation with an urban predator, while the opposite was observed when confronted with a rural predator. Patterns in handling times were similar, but less pronounced, and strongest in the thermal conditions mimicking urban conditions most (HW24-32°C, Fig. 2D). Both functional response parameters, here estimated based on the first three prey captured, culminated in a similar pattern for the overall predation rates: urban damselflies had higher predation rates compared to rural predators, but only when encountering rural water fleas, and not urban water fleas. In reverse, rural *Daphnia* were preyed upon more compared to urban *Daphnia* in the presence of urban predators, but not rural predators. Urban evolution in the prey

thus dampened the ecological effects of urban evolution in the predators. The combined effect of evolution in both predator and prey concealed the effect of evolution in each of the interactors, thereby generating an ecological feedback ('broad sense', Hendry 2017, De Meester et al. 2019) from evolution which is consistent with a scenario of cryptic eco-evolutionary dynamics.

Our study highlights local adaptation and an eco-evolutionary feedback in the context of urbanization. Our results are in line with observations along latitudinal thermal gradients, where striking interaction effects of the genetic background of damselfly predators and water flea prey, originating from different latitudes, were reported (De Block et al. 2013). Predation rates of low-latitude damselflies on low-latitude *Daphnia* were much higher than those of high-latitude damselflies on high-latitude *Daphnia*. We show that genotype  $\times$  genotype interactions in this classical predator-prey model system also occur across local thermal gradients, where dispersal-mediated mixing of predators and prey from different evolutionary backgrounds (i.e. urban–rural) are likely more frequent compared to mixing across latitudes. Notably, the here reported cryptic eco-evolutionary feedback is not negligible. Scenarios where one of the interacting species did not evolve in response to urbanization (e.g. as a consequence of reduced evolutionary potential or because of recent immigration from a different background) would lead up to a ca 250% and 25% increase in encounter and predation rates, respectively, compared to rural-rural and urban-urban conditions (Fig. 2C,I).

Compared to most other studies on eco-evolutionary feedbacks, we here worked with natural populations that evolved in situ under the natural selection regimes thereby working with realistic selection landscapes. Yet, further increasing realism during the predation trials would be an important next step. By having done the trials at one, ad libitum density, at a given light level and in the absence of submerged macrophytes, the absolute predation rates we observed may have been different compared to those in situ. Yet, it is important to note that these environmental factors (transparency, macrophyte cover, *Daphnia* density) do not systematically differ between urban and rural ponds in our study area (Engelen 2017). Thermal evolution likely is a driving factor for the observed evolutionary morphological, life history, and physiological trait differences (Brans et al. 2017b, 2018b, Tüzün et al. 2017, Tüzün and Stoks 2021), as well as for the here reported patterns in

predation rates – an ecologically relevant trait. It is therefore likely that running the predation trials under different environmental conditions in terms of light level, macrophyte presence or *Daphnia* densities would not change the patterns of relative differences in predation rates in function of the urbanization backgrounds of both predator and prey. Nevertheless, given the complexity of urban systems, we cannot fully exclude that other potential, non-temperature related, factors may contribute to the here observed responses.

Using a single prey density, we estimated the two functional response parameters directly from observations, instead of using the more extended method of measuring predation rates across prey densities and fitting a functional response model to estimate parameter values. Previous studies have reported that both methods can yield similar results, but can also differ depending on predator satiation level or density-mediated changes in predator behaviour (Novak and Wootton 2008, Sentis et al. 2013b). It would thus be interesting to conduct a similar study using the extended method to better understand the role of prey density in eco-evo feedbacks.

#### *Underlying mechanisms – evolutionary responses to urbanization*

The cryptic eco-evolutionary feedback reported here is linked to urban evolution of both interaction partners. Given that the damselfly families and *Daphnia* clones used in this study were isolated from different localities, it is highly unlikely that our findings are the result of direct co-evolution. Rather it is related to independent trait evolution to urban environments in interacting species. Urban evolution in both damselflies and in *Daphnia* is driven by, amongst others, the urban heat island effect (Brans et al. 2017b, 2018b, Tüzün et al. 2017, Tüzün and Stoks 2021). In the current study we did not observe that urban *Ischnura* damselflies evolved a higher heat tolerance compared to rural damselflies, yet they had a slower development and a higher body mass compared to rural damselflies (see Appendix – section B. Results), in line with earlier on a closely related species (*Coenagrion puella*, Tüzün et al. 2017). In contrast, the urban *Daphnia* used in the present study were smaller than rural ones, again in line with earlier work (Brans et al. 2017b, Brans and De Meester 2018). Urban evolution thus both involved a phenological mechanism (predator: countergradient variation as a consequence of longer

growing seasons in urban ponds, Tüzün et al. 2017, Brans et al. 2018b), and direct selection for warming tolerance (temperature-size rule, Daufresne et al. 2009, Brans et al. 2017b).

While body size of both predators and prey evolved in response to urbanization, it cannot explain the observed patterns, as our analyses included body mass (or size) of both species as a covariate. Rather, our results are potentially related to acquired energy reserves and activity levels. Earlier work has shown that urban *Daphnia* have higher levels of energy reserves (fat, protein, and carbohydrates, Brans et al. 2018a) and a faster pace-of-life (e.g. faster development rate, Brans and De Meester 2018) than rural *Daphnia*. These traits are likely linked to a higher movement and grazing activity to increase energy intake (Réale et al. 2010). Similarly, Tüzün & Stoks (2021) recently showed that immune-challenged urban damselfly nymphs (*C. puella*) exposed to simulated heatwaves suffered less from heatwave-induced depletions in energy availability compared to rural damselfly nymphs. This suggests that urban damselfly populations too have evolved bio-energetic compensatory mechanisms to better cope with heat stress in the city (Tüzün & Stoks, 2021), and these mechanisms might similarly be linked to a higher foraging activity. The combined research on this study systems tentatively suggests that both predators and prey have evolved changes in activity and feeding behavior in response to urbanization. While damselflies are commonly sit-and-wait predators, increased activity (e.g. walks, number of attacks, head turns) in urban populations could increase their predation efficiency and thus food intake in comparison to a less active rural predator (Gerritsen and Strickler 1977, Werner and Anholt 1993). In contrast, urban *Daphnia* that are more active and have more energy reserves can spend more time in faster longer distance ‘cruising’ behavior, compared to a rural prey, that might be confined to typical slower, local, vertical ‘hop-sink’ movements. While damselflies are usually sit-and-wait predators, even slight changes in activity patterns of both predator and prey may thus impact encounter rates and successful attack rates (Pawar et al. 2012, Dell et al. 2014), in such a way that they are higher between urban predators and rural prey and vice versa (one interactor is less spatially confined), compared to reduced encounter, and thus predation, rates when predators paired with prey from the same evolutionary background (urban-urban: both interactors are active, but elude each other; rural-rural: both interactors are less active and more spatially confined)

(McCauley et al. 1993). Note that while we cautiously proposed a number of plausible mechanisms related mainly to energy acquisition and activity patterns that could be driving the here observed patterns, these clearly need further detailed studies for confirmation.

#### *Conclusions & Future directions*

By explicitly taking a multispecies perspective on eco-evolutionary dynamics, carrying out predation trials in a common-garden setting in which we manipulated the genetic background of both predator and prey, we here document a unique example of a strong, yet cryptic evo-to-eco feedback. Notably, we revealed this cryptic feedback across a strong selection gradient associated with urbanization. Encounter and predation rates are similar for urban predators feeding on urban prey and for rural predators and rural prey, misleadingly suggesting no evolution occurred in urban ponds. Yet strong effects of evolution are revealed in combinations where only one of the interactors evolved in response to urbanization. Such cryptic eco-evolutionary interactions are important, as they imply that strong deviations from expectations might be occurring, here more than a doubling of predation rates, in case interacting species would not have the same opportunity to evolve (e.g. due to genetic constraints). Damselflies are efficient predators on *Daphnia*. As a result, mismatches in degree of urban evolution resulting in higher predation rates might potentially substantially reduce population sizes of *Daphnia*. Large-bodied *Daphnia*, in turn, are highly efficient grazers on algae compared to other zooplankton (Chislock et al. 2013, Gianuca et al. 2016). Reduced population sizes of large-bodied *Daphnia* might thus result in a strongly reduced top-down control of phytoplankton. Given the urban heat-island effect combined with nutrient enrichment favor the formation of (toxic) algal blooms (Waajen et al. 2014), such evolutionary mismatches may thus have important consequences. To inform the extent to which urban eco-evolutionary feedbacks dampen or amplify ecosystem-wide changes, such as algal blooms (Des Roches et al. 2020), we propose larger outdoor mesocosms or transplant experiments with fully crossed urban and rural predator-prey combinations, whereby more natural settings (e.g. predator and prey are interacting throughout the heatwave period) are better mimicked (Brans et al. 2020).

The here studied example and other cases of cryptic evo-to-eco feedbacks also illustrate why eco-evolutionary dynamics have often been overlooked in the past (Kinnison et al. 2015). Finally, our results demonstrate the importance of quantifying the effect of evolution of multiple species in communities when studying eco-evolutionary dynamics (De Meester et al. 2019). The effects of evolution in multiple species likely often will not simply add up, and might in some cases actually cancel each other out, as was observed in the present study. It is important to study whether and to what extent evolution of multiple species leads to additive, synergistic or compensatory effects on eco-evolutionary dynamics in other systems as it will improve our forecast on how communities of evolving and dispersing species will respond to the challenges of the Anthropocene (Urban et al. 2016).

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## **Conflict of interest**

Authors declare no competing interest.

**Author contributions statement:** KIB, NT, LDM and RS designed the study; NT and KIB collected the data; NT, KIB, and AS analyzed the data; KIB wrote the first draft of the manuscript and, together

with NT, led the writing of the manuscript and subsequent revisions. All authors contributed critically to the various drafts of the manuscript.

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## Tables

**Table 1.** Linear mixed-effect model results for (A) encounter rate ( $s^{-1}$ , log-transformed), (B) handling time (s, log-transformed), and (C) predation rate (ratio of  $N_{\text{eaten}}/N_{\text{initial}} \cdot h^{-1}$ ) in response to the heatwave treatment (HW; HW20-28°C, HW24-28°, HW24-32°C), and urbanization backgrounds (urban/rural) of both *Ischnura* predators and *Daphnia* prey. The time point of successful attack (first, second, third), prey body size (mm) was included as fixed effect for encounter rate and handling time. For all variables, sex of the predator was included as fixed effect and predator body mass (mg, corrected for number and mass of *Daphnia* eaten at the end of the trial) and prey size (mm) as covariates, as well as the random nested structure of *Daphnia* clone and *Ischnura* family (mother identity) nested in population, and population nested in urbanization category for both interactors. For all three variables heatwave date (i.e. date of the start of the heatwave) was added as random effect to the model.

<b>A. Encounter rate (<math>s^{-1}</math>)</b>	<b>NumDF</b>	<b>DenDF</b>	<b>F</b>	<b>P</b>
Heatwave treatment (HW)	2	127.2	2.289	0.105
Urbanization predator	1	7.0	0.930	0.367
Urbanization prey	1	9.6	0.014	0.909
HW $\times$ Urbanization predator	2	130.3	0.593	0.554
HW $\times$ Urbanization prey	2	131.3	0.149	0.862
Urbanization predator $\times$ Urbanization prey	1	131.6	16.111	<b>&lt;0.001</b>
HW $\times$ Urbanization predator $\times$ Urbanization prey	2	134.3	2.675	0.073
Time point attack	1	146.0	5.536	<b>0.020</b>
Sex predator	1	142.2	0.001	0.979
Prey ( <i>Daphnia</i> ) size (mm)	1	117.1	0.092	0.762
Predator ( <i>Ischnura</i> ) body mass (mg, corrected)	1	138.0	1.277	0.260
<b>B. Handling time (s)</b>	<b>NumDF</b>	<b>DenDF</b>	<b>F</b>	<b>P</b>
Heatwave treatment (HW)	2	142.6	0.078	0.925
Urbanization predator	1	143.9	0.894	0.346
Urbanization prey	1	146.0	0.024	0.877
HW $\times$ Urbanization predator	2	143.6	0.244	0.784
HW $\times$ Urbanization prey	2	145.2	3.558	<b>0.031</b>
Urbanization predator $\times$ Urbanization prey	1	145.9	0.232	0.631
HW $\times$ Urbanization predator $\times$ Urbanization prey	2	146.3	5.090	<b>0.007</b>
Time point attack	2	296.4	7.007	<b>0.001</b>
Sex predator	1	148.6	0.413	0.521
Prey ( <i>Daphnia</i> ) size (mm)	1	130.2	0.835	0.363
Predator ( <i>Ischnura</i> ) body mass (mg, corrected)	1	146.4	1.110	0.294
<b>C. Predation rate (<math>N_{\text{eaten}}/N_{\text{initial}} \cdot h^{-1}</math>)</b>	<b>NumDF</b>	<b>DenDF</b>	<b>F</b>	<b>P</b>
Heatwave treatment (HW)	2	106.8	7.079	<b>0.001</b>
Urbanization predator	1	21.3	2.701	0.115
Urbanization prey	1	23.1	1.597	0.219
HW $\times$ Urbanization predator	2	105.3	1.101	0.336
HW $\times$ Urbanization prey	2	111.8	1.531	0.221
Urbanization predator $\times$ Urbanization prey	1	121.3	5.663	<b>0.019</b>
HW $\times$ Urbanization predator $\times$ Urbanization prey	2	112.5	0.277	0.758
Sex predator	1	139.8	1.149	0.286
Prey ( <i>Daphnia</i> ) size (mm)	1	140.1	11.151	<b>0.001</b>
Predator ( <i>Ischnura</i> ) body mass (wet weight, mg)	1	142.2	0.704	0.403

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## Figure legends

**Figure 1.** Schematic representation of the experimental scheme for the predation trials. Both urban and rural *Ischnura* predators (left) and *Daphnia* prey (right) were reared at 20 and 24°C in the lab (rearing procedures are detailed in material and methods), and thereafter exposed to either one of three five-day simulated heatwave treatments (HW20-28°C, HW24-28°C, HW24-32°C), independently. On the final day of each simulated heatwave, one predator and 20 prey were combined in a predation trial during which functional response parameters (encounter rate, handling time, predation rate) were assessed. We tested all four possible combinations of predator and prey urbanization background at each of the two heatwave temperatures (each time matching predator and prey of the same HW treatment). For each predator background (urban/rural) × prey background (urban/rural) × heatwave treatment (HW20-28°C, HW24-28°C, HW24-32°C) combination 12 replicate trials were conducted.

**Figure 2.** (A, B) Encounter rate ( $s^{-1}$ ), (D, E) handling time (s), and (G, H) predation rates (number of *Daphnia* eaten / number of initial *Daphnia*.  $h^{-1}$ ) of urban and rural nymphal *Ischnura elegans* predators on urban and rural *Daphnia magna* prey for three different heatwave scenarios. Panels A-B, D-E and G-H: x-axis labels urbanization background of predator, bar colour depicts urbanization background of prey (blue: rural; red: urban). Panels B, E, H show response variables averaged across the heatwave scenarios to visualize cryptic eco-evolutionary feedbacks. Shown are least squares means  $\pm 1$  SE. Panels C, F, I depict percentage change in each response variable relative to trials with a rural predator and a rural prey (predator urbanization on x-axis; dots reflect predation trials with rural prey, triangles indicate predation trials with urban prey; full arrows: change upon mismatch between urbanization level of predator and prey; dashed line: change from rural-rural to urban-urban). Red pictograms of predator and prey refer to an urban evolutionary background; blue pictograms refer to predators and prey with a rural background. Urban evolution in *Daphnia* buffers for the observed urban evolution-driven changes in an ecological response (encounter and predation rates) in the

predator (significant interaction between urbanization background of the predator × urbanization background of the prey, Table 1). Comparing encounter and predation rates of urban vs. rural predators using their respective prey type (horizontal lines, panels B, H) would have masked urban evo-to-eco feedbacks (i.e. ‘cryptic eco-evo feedback’). Shown are least squares means  $\pm 1$  SE. Different letters above (encounter rate, handling time, panels A,D) or within (predation rate, panel G) bars denote significant differences in response variables based on post-hoc comparisons reported on in results).

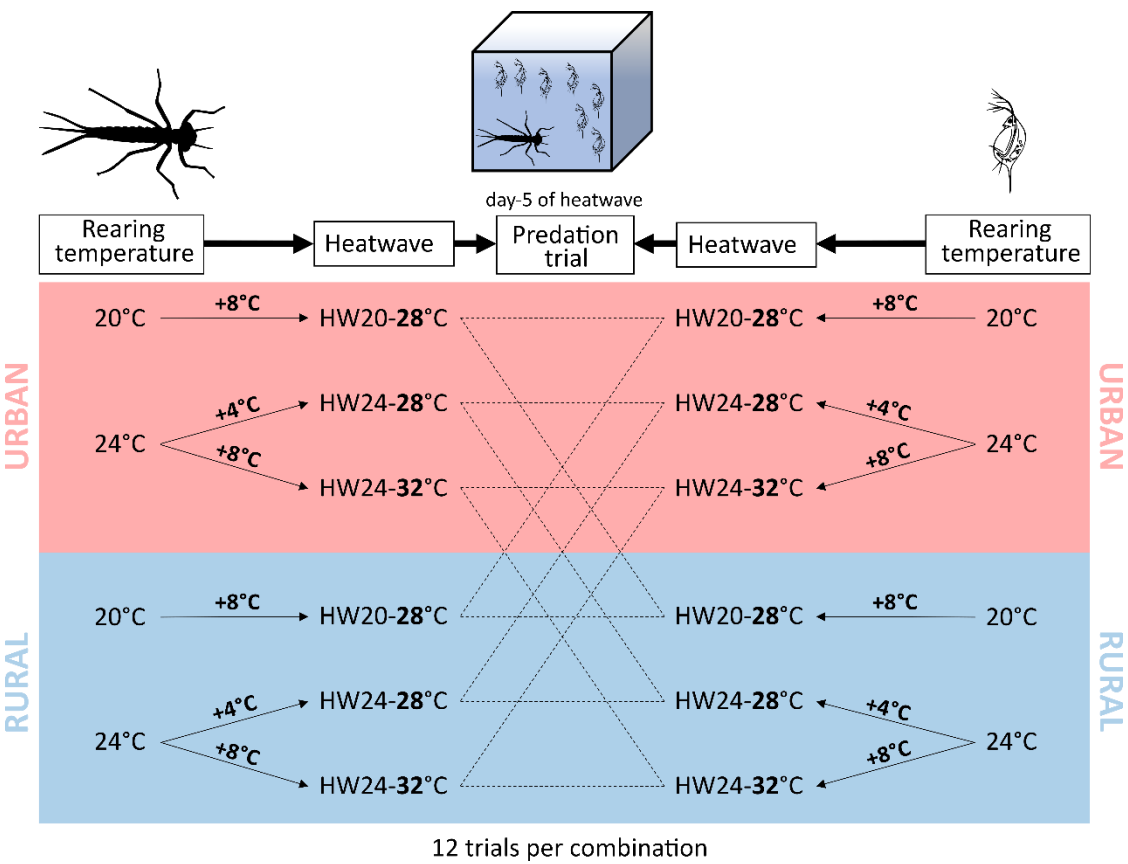


Figure 1.



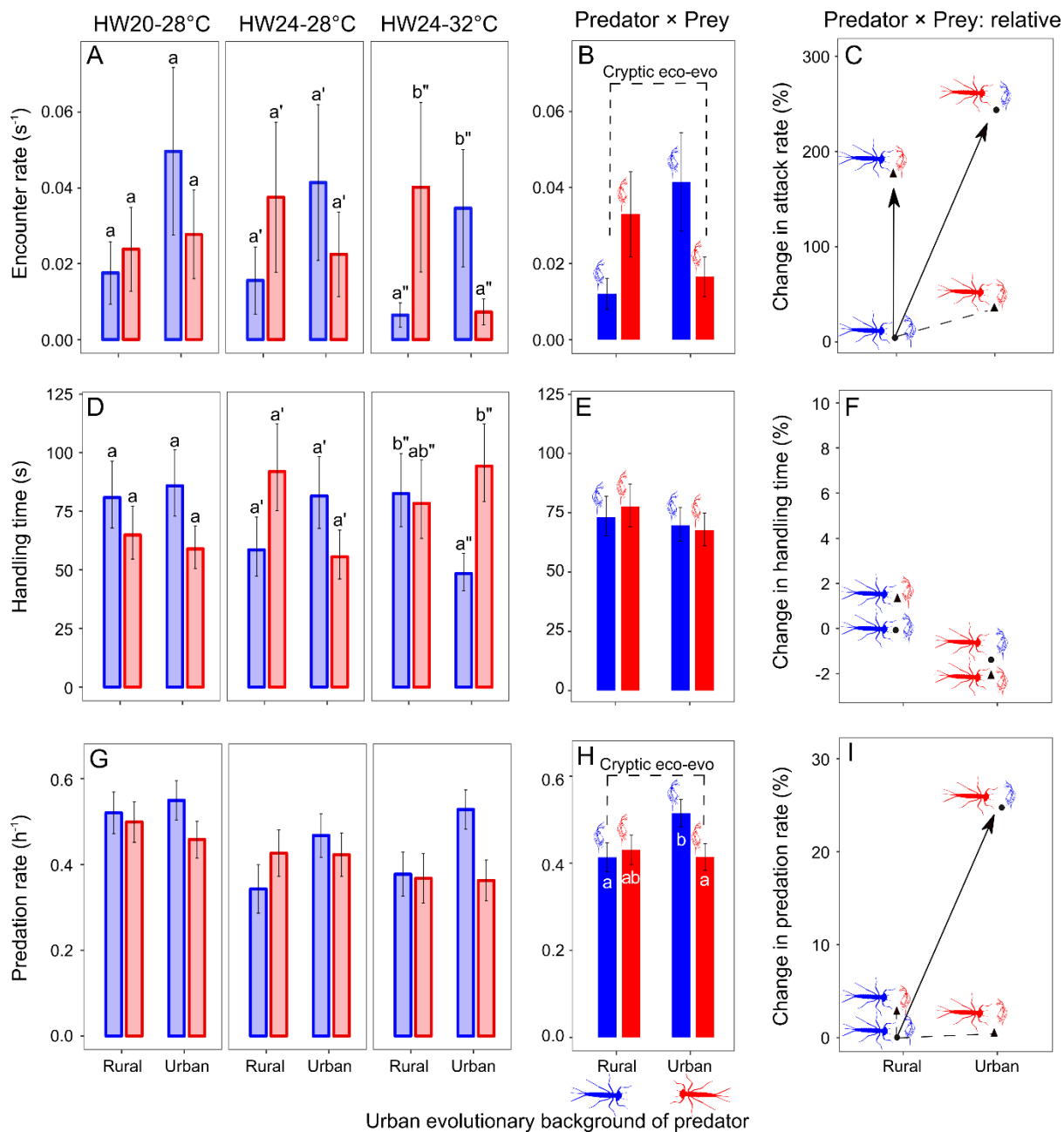


Figure 2.