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Research Article

Non-consumptive effects of native, alien and invasive alien crayfish on damselfly egg life history and carry-over effects on larval physiology

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Abstract

Invasive alien (IA) predators pose significant threats to native ecosystems, often leading to profound impacts on prey species through both direct and non-consumptive effects (NCE). This study focused on the NCE of predator-induced stress from one native crayfish species, noble (*Astacus astacus*), compared to one alien danube crayfish (*Pontastacus leptodactylus*) and two IA crayfish species, signal (*Pacifastacus leniusculus*) and spinycheek crayfish (*Faxonius limosus*), on the native damselfly *Ischnura elegans*. We investigated the direct crayfish cue effect on egg traits as well as potential carry-over effects from the egg stage to the larval stage. We hypothesised that native crayfish cues would lead to more pronounced negative effects on prey traits compared to alien and IA crayfish, due to an evolutionary history of interaction and recognition of these threats. Unexpectedly, compared to native crayfish cues, alien and IA crayfish cues caused significantly higher egg mortality and prolonged developmental times, particularly cues from danube and signal crayfish, while cues from spinycheek crayfish had weaker, yet, still significant effects. Hatching synchrony was reduced and this to the same extent by the cues of all four crayfish species. Notably, cues from both alien and IA crayfish species caused significant carry-over effects, resulting in reduced larval survival, mass and fat content, which were more pronounced for danube and signal crayfish. Native crayfish cues did not induce carry-over effects, suggesting that *I. elegans* may have evolved a degree of resilience against this predator or that native crayfish produce chemical cues that do not cause a strong antipredator response. Our findings underscore the importance of considering immediate and carry over effects of crayfish on prey traits across multiple life stages, particularly in the context of biological invasions.

Key words: Carry-over effect, invasive alien species, life history, phenotypic plasticity, physiology, predator-prey interaction

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Introduction

In natural systems, ecological stressors mediated by human activity such as the introduction of invasive alien (IA) predators can drastically affect native prey populations, cascading to changes in the dynamics of native prey communities (Sih et al. 2010; Bucciarelli et al. 2019). This can be explained by the fact that native prey lack evolutionary history with newly-introduced predators and do not recognise the predators as being dangerous (Schlaepfer et al. 2005; Anton et al. 2020). According to the “naïve prey hypothesis”, IA predators are expected to be more effective than native predators in preying upon local prey (Cox and Lima 2006). Although some studies supported this hypothesis (Juliano and Gravel 2002; Freeman and Byers 2006; Siesa et al. 2014), others did not (Antoł and Sniegula 2021; Amer et al. 2024). Hence, the importance of studying to what degree and in what direction IA predators differ from native predators in shaping local prey populations.

Predators can affect prey through direct consumptive effects or indirect non-consumptive effects (NCE). Predators can impose NCE by visual and/or chemical cues (kairomones), with the latter being unintentionally synthesised and released to the environment (Brown et al. 1970). By definition, kairomones evoke adaptive responses in prey (the receiver), increasing their chances of survival, while the release of these cues is maladaptive for the predator (Ruther et al. 2002). NCE might reduce prey population sizes to the same degree or, in some cases, to a higher degree than consumptive effects (Preisser et al. 2005; Preisser and Bolnick 2008; McCauley et al. 2011; Cinel et al. 2020; Sheriff et al. 2020). The NCE reduce prey fitness through risk-induced trait responses which affect prey fitness components, including behaviour, life history and physiology (Janssens and Stoks 2013; Garcia et al. 2017; Sniegula et al. 2019; Cinel et al. 2020; Yli-Renko et al. 2022; Wos et al. 2024). Although kairomones are often considered non-species-specific (Von Elert and Pohnert 2000), prey can respond differently to cues from various predator species, suggesting that the identity and composition of predator-released cues may vary (Turner et al. 1999; Van Buskirk 2001; Amer et al. 2024). Such variation may explain why prey exhibit different responses to alien predators that are chemically distinct from native species (Anton et al. 2020).

Kairomones are typically considered the primary drivers of NCE; however, predators can also host epibionts or promote microbial growth that may indirectly affect prey condition and survival, especially in aquatic systems (Ringelberg and Van Gool 1998; Dražina et al. 2018; Kumar et al. 2022). Such interactions highlight the multifaceted nature of predator-prey dynamics, suggesting that the effects observed may arise from a combination of chemical cues release by predators and biological contamination from epibionts.

While predator-induced changes in life history traits are well documented, effects of predators on physiological traits are less studied, yet also widespread (Hawlena and Schmitz 2010). While these may align and even explain effects on life history, they may also be decoupled (Slos et al. 2009; Raczyński et al. 2022) and indicate independent fitness-related effects that may become obvious only in the presence of other stressors. For example, NCE may reduce the prey energy content, thereby reducing their ability to deal with food stress and impair their immune and defence functions, thereby reducing the ability to deal with parasites, pathogens and toxic compounds (Stoks et al. 2006; Adamo 2022; Antoł et al. 2022).

NCEs might differentially affect prey traits during egg, larval and adult stages in prey with a complex life cycle (Sniegula et al. 2020; Amer et al. 2024; Supekar and Gramapurohit 2024). One reason is that different life stages typically differ in their risk of being preyed upon. Moreover, prey traits affected by NCE via exposure in a particular life stage can be coupled or decoupled with the following life stage. The coupling effect is assumed to be a result of latent or carry-over effects that link environmental stress in one life stage and phenotypic responses exhibited in later stages (Stoks and Córdoba-Aguilar 2012; Moore and Martin 2020). Nevertheless, also decoupling across life stages has been hypothesised (Moran 1994). There is mixed empirical evidence supporting these alternative hypotheses. For example, negative effects of predation risk experienced during the larval stage carried over to negatively affect adult mass and energy storage in a damselfly (Stoks et al. 2006), but no such carry-over effect of predation risk experienced in the egg stage affected subsequent survival until emergence in mosquitoes (Fontana-Bria et al. 2017).

In the studies on carry-over effects in general and especially for those related to predation risk, the egg stage has been understudied and typically only a limited number of egg traits have been considered. This may result in unmeasured or hidden carry-over effects of exposure to predation risk from the egg stage. The egg stage is a key window where the consequences of individual experiences can have lifelong effects on behaviour, physiology and fitness (Kingsolver et al. 2011), as shown in several studies (Chivers et al. 2001; Sniegula et al. 2017, 2019). Exposure to predation risk may directly affect the egg stage by, for example, shortening or prolonging the egg development time (Blaustein 1997; Anderson and Brown 2009; Fontana-Bria et al. 2017; Sniegula et al. 2019; Amer et al. 2024) or changing the synchrony of hatching (Bozelli et al. 2008). What is more, the propensity of egg exposure to predation risk to carry over to larval performance may vary across predator species (Sih and Moore 1993; Antoł and Sniegula 2021; Amer et al. 2024), ecology (Bucciarelli et al. 2019) and invasion history of IA predators at a local scale (Anton et al. 2020; Mathers et al. 2022).

Here, we compare the NCEs imposed by native, alien and IA opportunistic omnivorous crayfish species (Kozák et al. 2015) on key damselfly egg life history and larval physiological traits in central Europe. This is an interesting predator-prey study system to address this topic as there is ample natural history information on both the crayfish predators (Twardochleb et al. 2013; Pacioglu et al. 2020) and the damselfly prey (Cordoba-Aguilar et al. 2022) and there is a well-resolved phylogeny (Crandall and De Grave 2017) and invasion history of the crayfish predators at a regional and local scale (Grabowski and Jażdżewski 2005; Śmietana 2011a, 2011b; Kouba et al. 2014). Previous studies indicated that the aquatic stages of damselflies can react to both native and invasive crayfish species in their life history traits (Siesa et al. 2014; Antoł and Sniegula 2021; Antoł et al. 2022; Palomar et al. 2023; Amer et al. 2024) and these responses were evident even at the level of gene expression (Wos et al. 2024). Yet, it is not clear whether such responses carry over across developmental stages and are present at the physiological level, which would broaden our understanding of the potential impact of exposure to alien and IA crayfish on natural prey populations.

Here, we focus on two fitness-related types of physiological traits, investment in immune function and energy storage, which have both been shown to be sensitive to predation risk in damselfly larvae (e.g. Stoks et al. (2006) Van Dievel et al. (2016)). Based on the naïve prey hypothesis (Cox and Lima 2006), we predicted (1A) the strongest effects on damselfly egg traits, i.e. egg development time until hatching, hatching synchrony and survival under the NCE of native crayfish species, weaker NCE of alien and IA crayfish species that has already invaded damselfly sites for several decades and the weakest or no NCE of IA crayfish species that has not yet invaded the damselfly sites. We further test whether exposure to native, alien and IA crayfish-associated chemical cues (CACC) in the egg stage generates carry-over effects into the larval stage. We predicted that (1B) exposure of eggs to

CACC released by native crayfish species will have negative carry over effect on larval mass, energy storage (measured as fat content) and investment in immune function measured as phenoloxidase activity, whereas exposure of eggs to CACC released by alien and IA crayfish will have weaker or no effects on larval traits because of damselfly naivety. Alternatively, alien and IA crayfish will cause (2A) stronger effects on egg traits and (2B) carry over effects on larval traits due to the absence of evolutionary exposure of prey to these predators (Sih et al. 2010; Anton et al. 2020; Antoł and Sniegula 2021; Amer et al. 2024), hence the opposite of the naïve prey hypothesis. Finally, the equally valid alternative hypothesis states that (3) the intensity of responses to CACC in the egg stage and carry-over effects in the larval stage will be based solely on predator phylogeny with relation to native crayfish species. Specifically, we expect that the closer the predator species' relatedness, the more similar the damselfly's response will be, due to the similar chemical composition of CACC (Anton et al. 2020).

Methods

Background

Ischnura elegans is one of the most common native damselfly species in central Europe. It lives in a variety of freshwater habitats, including lentic and lotic waterbodies (Dijkstra and Schröter 2020). Adult females commonly deposit eggs into decaying aquatic plants that flow on the water surface. The juvenile aquatic stage shares habitats with several top predator species (Corbet 1999), including fish and crayfish (Schaffner and Anholt 1998; Le Gall et al. 2017; Sniegula et al. 2019; Palomar et al. 2023). Both egg and larval stages react to these predator cues (Antoł and Sniegula 2021; Wos et al. 2023; Amer et al. 2024; Sniegula et al. 2024). Geographic dispersal and high gene flow, particularly at the local scale, have been documented in *I. elegans* (Babik et al. 2023) and this factor might contribute to the damselfly response to alternative predator species and types (i.e. native vs. IA).

We studied two crayfish species that are native to Europe: the noble crayfish (*Astacus astacus*) and the danube crayfish (*Pontastacus leptodactylus*). The noble crayfish species is listed as vulnerable in Europe on the IUCN Red List (Gherardi and Souty-Grosset 2010). It is protected by law in Poland, though its population numbers decline (Krzywosz and Śmietana 2004; Bonk et al. 2014; Stanek et al. 2015; Rozporządzenie Ministra Środowiska 2016). The danube crayfish originates from the Caspian Sea region and was introduced to central Europe in 19th century. Although the species is considered native to Europe, it is classified as alien in Poland. This is explained by the fact that the danube crayfish is alien for the Wisła and the Odra river drainages, covering the majority of Poland (Grabowski and Jażdżewski 2005; Kouba et al. 2014). Danube crayfish is one of the rarest and irregularly spread crayfish in Poland, which supports its non-invasiveness. The species is listed as least concern on the IUCN Red List (Gherardi and Souty-Grosset 2010) and is protected by law in Poland (Rozporządzenie Ministra Środowiska 2016). Both noble and danube crayfish species occupy ponds and rivers close to the damselfly sampling sites (Bonk M, unpublished data; Strużyński 2007) (Fig. 1).

The two studied IA crayfish species were the spinycheek crayfish (*Faxonius limosus*) and the signal crayfish (*Pacifastacus leniusculus*), which are both native to North America. The spinycheek species has been introduced to central Europe at

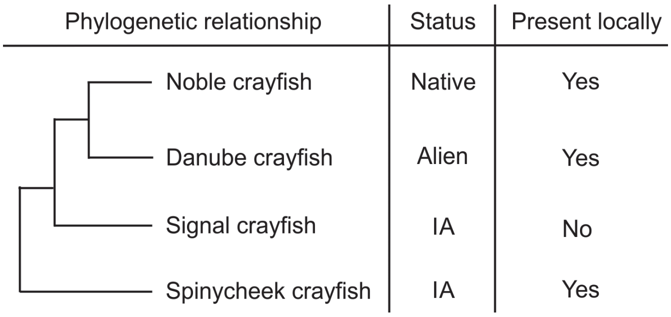

Figure 1. Phylogenetic relationships amongst the studied crayfish species (modified from Crandall and De Grave 2017), their invasive status and presence at the local scale in Poland.

the end of 19th century and is currently the most common crayfish in EU countries, including Poland and the study region specifically (Śmietana 2011a; Kouba et al. 2014; World of Crayfish™ 2024). It occupies ponds and rivers close to the damselfly sampling sites (Fig. 1). It is noted as one of the most ferocious invasive invertebrates that causes decline of native species, including both prey and native crayfish populations (Nentwig et al. 2018) (Fig. 1). The signal crayfish has been introduced to northern Europe (Scandinavia) in the 1960s and, since then, it has spread across most of European countries (Śmietana 2011b). In Poland, the crayfish is found mainly in northern regions, but is expected to invade southern Poland, i.e. the damselfly sampling sites, in the near future; in 2020, new sites were found ca. 200 km west from where the damselflies have been collected (Barowska et al. 2023) (Fig. 1).

Phylogenetically, noble and danube crayfish are sister taxa and signal crayfish is from the same clade. In contrast, the spinycheek represents a different family and at the phylogenetic level is equally distanced from noble/danube and signal crayfish (Crandall and De Grave 2017) (Fig. 1).

Animal collection and rearing

Copulating adult female *Ischnura elegans* were collected using a butterfly net on 15 June 2021 from two nearby ponds in the city of Krakow, Poland: Mydlniki ponds (50°05'09.6"N, 19°50'21.8"E) and Bonarka pond (50°01'25.4"N, 19°57'06.5"E). We selected these ponds because they supported numerous *I. elegans* populations. Additionally, the availability of historical and current crayfish distribution data allowed us to explore possible effects of pond-specific history of crayfish and population differences in damselfly responses to chemical cues. Mydlniki ponds are sourced by the Rudawa River that holds native noble crayfish (A. Klaczak 2023, pers. comm). To our knowledge, no crayfish have been recorded in Bonarka pond. However, in a nearby pond (approx. 350 m away), the danube crayfish was recorded until 2019 (M. Bonk 2019, unpublished). This absence of crayfish in Bonarka pond suggests that damselflies from this site may not have co-evolved with crayfish predators, potentially resulting in increased stress responses to both native, alien and IA species. The uncertainty regarding crayfish presence in Mydlniki ponds might also contribute to naïve responses. On the other hand, strong gene flow between *I. elegans* populations, as shown in recent studies (Babik et al. 2023), could homogenise damselfly responses and limit local adaptations to specific predator types.

Field-collected female damselflies were put in plastic jars with moisturised filter paper for egg laying and transported by car to the laboratory at the Institute of Nature Conservation PAS in Krakow. Jars with females were placed in a room with a temperature of 22 °C and natural day light. In total, 19 females from Mydlniki pond and 12 females from Bonarka pond laid large (> 100 eggs/clutch) egg clutches between 16 and 17 June 2021. These clutches were used in the experiment.

All crayfish species were collected in the field and transported by car to the laboratory several weeks prior the start of the experiment. Noble and danube crayfish were collected from a private pond near the town Miejska Górka (51°39'13.2"N, 16°58'52.3"E), spinycheek crayfish were collected from an excavation pond in Kryspinów (50°02'56.8"N, 19°47'28.7"E) and signal crayfish were collected from Hańcza Lake (54°15'31.9"N, 22°48'51.9"E). Noble, spinycheek and signal crayfish were collected and housed with permissions from, respectively, General Directorate of Environmental Protection in Warsaw (per. DZP-WG.6401.147.2021.TŁ), Regional Directorate of Environmental Protection in Krakow (per. OP.672.4.2021.GZ) and Regional Directorate of Environmental Protection in Białystok and Krakow (per. WPN.6205.21.2020.ML and OP-I.672.8.2020.MK1).

The densities of crayfish in aquaria were based on the basal metabolic rate equations obtained for crayfish (Wheatly 1989). After weighing, we kept two specimens of noble, danube and signal crayfish (wet mass ca. 100 g for each species) and five specimens of spinycheek crayfish (wet mass ca. 100 g) per experimental aquarium. Crayfish were fed with fish food pellets twice per week and live chironomid larvae once per week.

At egg laying, every clutch (= family) was divided into five treatment groups, with 20 eggs per family per treatment. At hatching, these five egg-treatment groups were further split into two larval-treatment subgroups: a control group or a crayfish-exposure group. In the control subgroup, larvae were not exposed to CACC, allowing us to test for carry-over effects of predator exposure during the egg stage. Larvae in the CACC subgroup received the same crayfish treatment as in the egg stage. This resulted in nine treatment groups: control(egg) – control(larva), noble(egg) – control(larva), noble(egg) – noble(larva), danube(egg) – control(larva), danube(egg) – danube(larva), spinycheek(egg) – control(larva), spinycheek(egg) – spinycheek(larva), signal(egg) – control(larva) and signal(egg) – signal(larva) group (Fig. 2). Throughout the experiment individuals were followed at the family level. Eggs were moved to separate 200 ml drinking cups (height – 9 cm, depth – 4 cm) creating sets of 20 eggs/cup. Accidentally, 15 out of 142 cups contained more than 20 eggs, which was accounted for in the statistical analysis by including egg density as a covariate in our models. Every cup was filled with 67 ml of dechlorinated tap water and 33 ml of treatment water with or without CACCs. To introduce the CACCs, we used water from the aquaria holding crayfish. As a control, we used dechlorinated tap water held in the same type of aquarium as the aquaria with crayfish. We placed cups with eggs in an incubator (ST700, Pol-Eko) at a constant temperature of 20 °C and a photoperiod of L:D 16:8 h. The cups were randomly distributed to the treatments. We replaced 33 ml of water in cups with water from the appropriate crayfish species and control aquarium every second day. The median half-life of predator cues is ca. 48 h (Van Buskirk et al. 2014). The effectiveness of the here-applied CACC refill frequency has been confirmed in previous experiments on damselfly eggs and larvae (Sniegula et al. 2019; Raczyński et al. 2022; Amer et al. 2024).

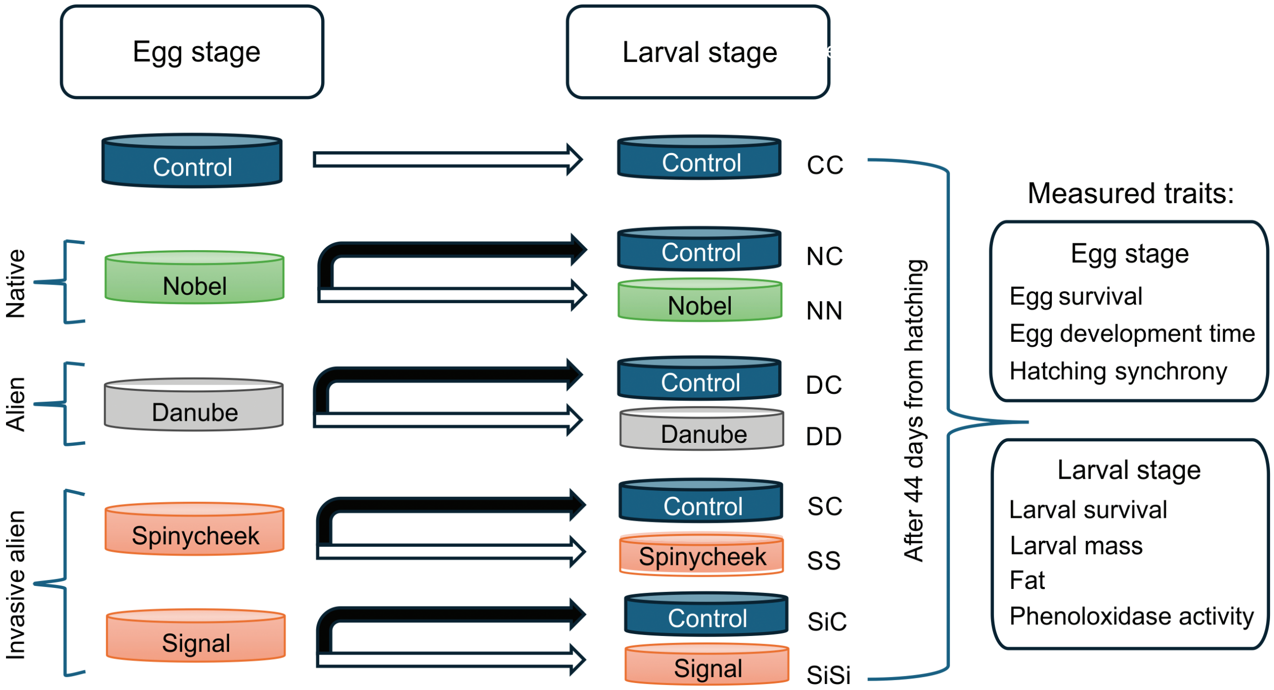

Figure 2. A scheme of the experimental method, showing egg and larval crayfish treatments and the traits measured 44 days after hatching. Filled arrows indicate carry-over non-consumptive effect (NCE), empty arrows indicate continues exposition to NCE. Abbreviations for the crayfish treatment groups are indicated to the right of the larval treatment groups: CC – control(egg)-control(larva), NC – noble(egg)-control(larva), NN – noble(egg)-noble(larva), DC – danube(egg)-control(larva), DD – danube(egg)-danube(larva), SC – spinycheek(egg)-control(larva), SS – spinycheek(egg)-spinycheek(larva), SiC – signal(egg)-control(larva) and SiSi – signal(egg)-signal(larva) group.

The number of larvae hatched per cup ranged from 2 to 34. The larvae were fed *ad libitum* daily with laboratory-cultured *Artemia* nauplii. When the earliest hatched larvae in each cup reached the age of 44 days, all larvae from the same cup were group-weighed and frozen in the same Eppendorf tube at -80 °C for physiological analyses. We chose this larval age for two reasons: it represented approximately 50% of the larval development time until emergence and each group had reached the minimal wet mass threshold for the analysis of physiological traits.

Response variables

The proportion of eggs that survived per cup was calculated as the number of eggs per cup that hatched. The unhatched eggs were considered as dead. We noted the egg development time from egg laying to hatching. Every cup was checked for new hatchlings every morning and afternoon, with half a day used as the measurement unit. Based on the egg development times in a given cup, we estimated hatching synchrony per cup as the coefficient of variation (CV); the smaller the CV, the higher the hatching synchrony. This trait is relevant to measure because it can represent one of the preys' tactics for escaping predation pressure, for example, predator satiation effect (Janzen 1971) or bet hedging tactic (Simons 2011). Larval survival was measured as the number of larvae per cup that survived until day 44 after the first individual in the cup hatched. Mean larval wet mass per cup was measured when the first larva in that cup reached the age of 44 days after hatching and was calculated as the total mass divided by the number of larvae per cup (1–8 larvae per cup).

Physiological traits

We assessed physiological traits from the body supernatants of preserved larvae. To prepare the body supernatant, the larvae were homogenised in PBS buffer (Phosphate-Buffered Saline, final mass × 15 µl PBS) and subsequently centrifuged.

As a measure of investment in immune function, we quantified the activity of phenoloxidase (PO). This enzyme plays a key role in the defence of insects against bacterial, fungal and viral agents (González-Santoyo and Córdoba-Aguilar 2012). The PO activity assay followed the method described by Stoks et al. (2006). In this assay, 10 µl of the homogenate was combined with 105 µl of phosphate-buffered saline (PBS) and 5 µl of chymotrypsin and the mixture was incubated for 5 minutes in a 384-well microtiter plate. Subsequently, L-DOPA (1.966 mg dihydroxyphenyl-L-alanine per 1 ml of PBS buffer) was added to the samples. The linear increase in absorbance at 490 nm was measured every 20 seconds for 30 minutes at 30 °C. The average of the duplicate readings for each sample was used for statistical analyses. PO activity was expressed in nmol of dopachrome formed per minute. To normalise PO activity, the protein content in the supernatant of each sample was measured using the Bradford method (Bradford 1976).

We determined the fat content of damselfly larvae using a modified protocol based on Marsh and Weinstein (Marsh and Weinstein 1966), as described by Verheyen et al. (Verheyen et al. 2018). Small glass tubes were filled with 8 µl of supernatant and 56 µl of concentrated sulphuric acid (100%). The tubes were heated at 150 °C for 20 minutes, then allowed to cool before adding 64 µl of milliQ water. A 380-well microtiter plate was loaded with 30 µl of the final mixture per larva in triplicate and absorbance was measured at 490 nm. The mean of the three readings was used for statistical analyses.

Statistical analyses

All the tests were performed using R version 4.3.2. Following packages were used: the lme4 package for general linear mixed models (Bates et al. 2015), the car package for estimating p-values (Fox and Weisberg 2019) and the *summary* function for checking contrasts between different levels (specifically, between control and different CACC treatments and between ponds). For the graphics, the ggplot2 package was used (Wickham 2016). We assessed the homogeneity of variance and the normality of residuals by visually examining the residual plots. In a separate analysis for egg and larval survival, proportions of surviving eggs or larvae per cup were response variables (both arcsin transformed) and CACC treatment (five levels for the egg stage and nine levels for the larval stage) and pond (two levels) were explanatory variables. Similar tests, but with no transformation of response variables were used for analysing the hatching synchrony, egg development time, mean larval mass per cup, mean fat storage per cup and mean PO activity per cup. As, at the end of the experiment, cups held different number of larvae, analyses of larval mass, total fat content and PO activity per cup were corrected by the number of larvae per cup. In all models, family nested in pond was added as random effect. We initially fitted global models that incorporated all main effects and interaction terms. Interaction terms with p-values greater than 0.05 were then excluded from the final models.

Results

The number of individuals considered in the analyses ranged from 86 to 339 (egg treatments) and from 5 to 10 (cumulative egg and larval treatments) per treatment combination. Suppl. material 1: table S1 shows the number of individuals across all treatment groups.

The analyses showed significant main effects of CACC on egg and larval life history and physiological traits, as well as interacting effect of CACCs and pond on egg life history in *I. elegans*.

Family (random effect) explained 21.5% of the variance in egg development time and 17.7% in larval mass. Variance explained by family was lower for other traits and models for hatching synchrony and larval fat content indicated negligible family-level variance (model singularity). The variance explained by family was generally higher after accounting for fixed effects, such as CACC treatment and pond, which indicated the role of experimental treatments in shaping observed variability. A table summarising the variance explained by family is available in Suppl. material 1: table S2.

Effects during the egg stage

In general, CACC had a negative effect on egg survival (Fig. 3A, Table 1). This significant result was mainly caused by the signal CACC, which decreased egg survival by half and the danube CACC, which decreased egg survival by a fourth compared to the control group. Noble and spinycheek CACC did not affect egg survival (Fig. 3A, Suppl. material 1: table S3). The two pond populations did not differ in egg survival (Fig. 3A, Table 1).

Overall, eggs took longer to develop under the CACC treatment. This result was especially pronounced under the signal CACC (+10 days), which caused the longest egg development time, followed by the danube (+7 days), spinycheek (+4 days) and noble (+2 days) CACC. These results were supported by Tukey's HSD pairwise comparisons (Fig. 3B, Suppl. material 1: table S4). The significant interaction between CACC and pond indicated that the effect of signal CACC cue (compared to the pond control) is stronger in Mydlniki pond than in Bonarka pond. Yet, the ponds did not differ from each other for a given CACC treatment (Suppl. material 1: fig. S1, table S4; Table 1).

Hatching was about two times more synchronised under the control treatment than in the presence of CACC (Fig. 3C, Table 1). The hatching synchrony did not differ between any of the treatments with CACCs (Suppl. material 1: table S5). Ponds did not differ in hatching synchrony (Fig. 3C, Table 1).

Effects during the larval stage

In general, exposure to CACCs decreased larval survival when quantified when the first larva in a cup reached an age of 44 days (Fig. 4A, Table 2). The decreased larval survival only occurred in response to danube and signal CACC and this both under combined egg-larval exposure (danube-danube and signal-signal CACC treatments) and under exposure of only the eggs (danube-control and signal-control CACC treatments), the latter indicating carry-over effects. In contrast, survival was not affected by exposure to noble and spinycheek CACC (noble-control, noble-noble, spinycheek-control and spinycheek-spinycheek CACCs) (Fig. 4A, Suppl. material 1: table S6). Damselfly larvae of both ponds did not differ in survival across all treatments (Table 2).

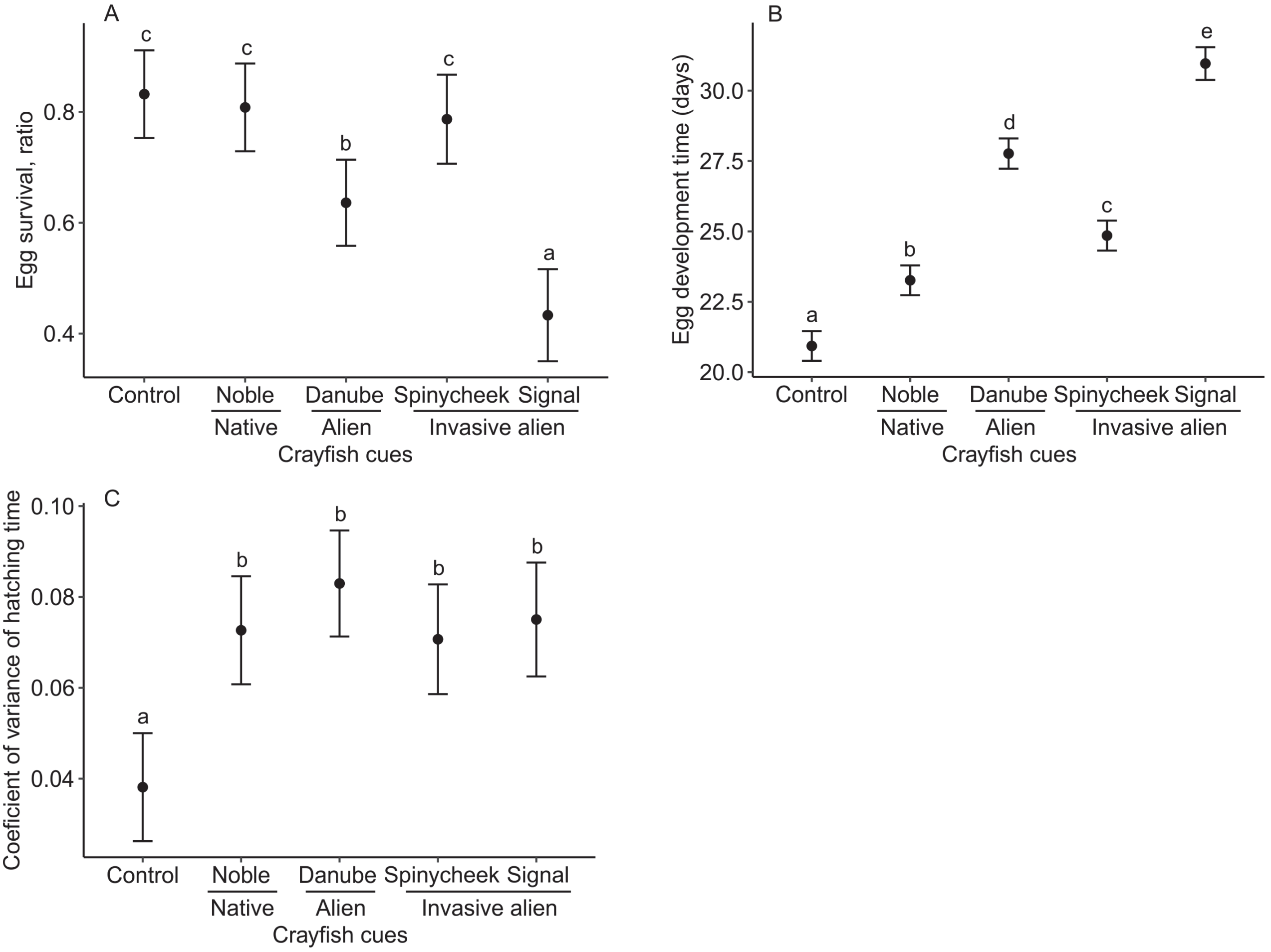

Figure 3. Effects of crayfish cues from native and invasive alien (IA) crayfish species on the egg survival rate (**A**), development time (**B**) and hatching synchrony (**C**) in *I. elegans*. Shown are means with 95% CI. Different letters indicate means that are significantly different, based on Tukey pairwise tests.

Table 1. Effects of crayfish-associated chemical cues (CACCs), pond and their interactions on egg survival, development time in days and hatching synchrony measured as coefficient of variation (CV) in *I. elegans*. Significant p-values are in bold.

Predictor	df	Chisq	p-value
Egg survival			
CACCs	4	78.8	**< 0.001**
Pond	1	0.2	0.680
Egg development time			
CACCs	4	1206.8	**< 0.001**
Pond	1	0.9	0.327
CACCs × pond	4	10.8	**0.028**
Hatching synchrony, CV			
CACCs	4	33.6	**< 0.001**
Pond	1	0.1	0.814

CACC decreased larval mass (Fig. 4B, Table 2). This mass decrease was especially pronounced under combined egg and larval exposure in the noble-noble, danube-danube and signal-signal CACC treatments and less so, but still significantly, under only egg exposure in the danube-control CACC treatment, indicating a carry-over effect. Exposure to spinycheek CACC never affected larval mass

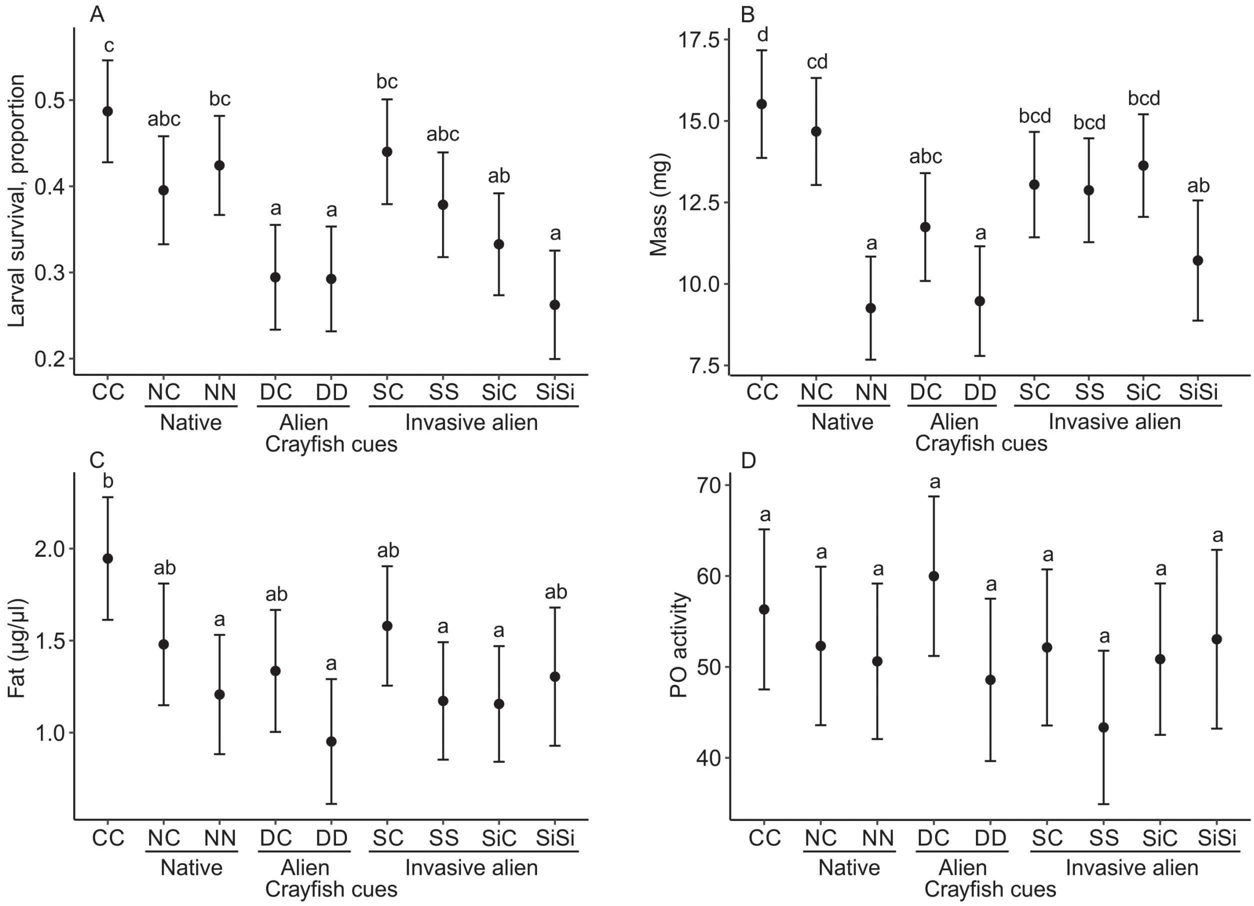

Figure 4. Effects of crayfish-associated chemical cues (CACCs) from native and invasive alien (IA) crayfish on the larval survival rate (**A**), mass (**B**), fat content (**C**) and phenoloxidase activity (D) in *I. elegans*. Note that in treatment combinations where the second letter is "C" (hence NC, DC, SC and SiC), the larvae were only exposed to the CACCs in the egg stage, but not in the larval stage, hence, when different from the control CC treatment would indicate a carry-over effect from egg exposure. Shown are means with 95% CI. Different letters indicate means that are significantly different, based on Tukey pairwise tests. Abbreviations for CACCs along the x-axis are as in Fig. 2.

Table 2. Effects of crayfish-associated chemical cues and pond on larval survival and mean values of mass, fat content and phenoloxidase activity (PO) per cup. Analyses on larval mass, fat content and PO were corrected by number of larvae per cup. Significant p-values are in bold.

Predictor	df	Chisq	p-value
Survival			
CACCs	8	56.7	**< 0.001**
Pond	1	0.2	0.675
Mass			
CACCs	8	67.6	**< 0.001**
Pond	1	0.1	0.732
No. of larvae per cup	1	175.6	**< 0.001**
Fat content			
CACCs	8	22.3	**0.004**
Pond	1	1.1	0.299
No. of larvae per cup	1	2.7	0.102
Phenoloxidase activity			
CACCs	8	10.9	0.211
Pond	1	0.3	0.592
No. of larvae per cup	1	31.4	**< 0.001**

(spinycheek-spinycheek, spinycheek-control) (Fig. 4B, Suppl. material 1: table S7). Damselfly larvae of both ponds did not differ in mass across all treatments (Table 2).

CACC negatively affected the total fat content (Fig. 4C, Table 2). The fat content decrease was especially pronounced under combined egg and larval exposure in the noble-noble, danube-danube and spinycheek-spinycheek CACC treatment, with the exception of the signal-signal CACC treatment where the effect was absent. Under only egg exposure treatment, the only significant effect was found under signal-control CACC treatment, indicating a carry-over effect (Suppl. material 1: table S8). Damselfly larvae of both ponds did not differ in fat content across all treatment groups (Table 2).

CACC did not affect phenoloxidase activity (PO) (Fig. 4D, Table 2), which was also supported in a Tukey's HSD pairwise comparisons (Suppl. material 1: table S9). Damselfly larvae of both ponds did not differ in PO across all treatments (Fig. 4D, Table 2).

Discussion

We examined the non-consumptive effects (NCEs) of native, alien and invasive alien (IA) crayfish species on the egg and larval traits of the damselfly *I. elegans*, with special attention for potential carry-over effects from the egg to the larval stage. Our results do not support the first (prey naivety) hypothesis stating that alien and IA crayfish species exert weaker effects on damselfly egg survival and development time than native crayfish species. Instead, the strongest effects were observed in response to the IA signal crayfish-associated chemical cues (CACCs), which has not yet invaded the damselfly sampling ponds, supporting one of our alternative hypotheses. Eggs exposed to these cues exhibited the longest development time, lowest survival and disrupted hatching synchrony. While we did detect negative carry-over effects on larval survival and mass in response to egg exposure to CACCs, this was only the case for cues from one alien crayfish (danube) and one IA crayfish (signal), supporting the alternative hypothesis (i.e. the opposite of the prey naivety hypothesis). Interestingly, only egg exposure to IA signal CACCs induced a negative carry-over effect on larval fat content. These are important results for invasion biology as they suggest that crayfish-induced NCE cannot only persist across life stages, but are also not restricted to native predators. Finally, there was limited support for the last alternative hypothesis, as the observed responses did not strictly follow phylogenetic relationships. In other words, closely-related crayfish species did not consistently elicit similar prey responses across all traits assessed.

Immediate NCE effects on the egg traits

Our findings underscore the importance of studying egg-stage predator-prey interactions in species with complex life cycles, as exposure during the egg stage can significantly influence fitness-related traits. CACC from the IA signal crayfish reduced by half egg survival and extended the egg development time by 10 days, indicating that the mere presence of IA predator-associated chemical cues can induce strong stress responses in damselfly eggs. Such responses are consistent with other studies demonstrating that exposure to predator cues during the early life stages can trigger significant physiological changes that decrease egg survival (Blaustein 1997; Miner et al. 2010; Sniegula et al. 2019). However, the reduction in egg survival does not align with the classical definition of kairomones, which indicates that prey responses should be adaptive (Ruther et al. 2002). In this case, the CACCs appear maladaptive for the prey, as the observed mortality does not confer any immediate survival benefit.

Interestingly, CACC from the alien danube crayfish, which has been present in the region for over a century (Strużyński 2007), also reduced egg survival and extended development time, though to a lesser degree. This happened despite the fact that the danube crayfish is known for being mild, relatively less active when feeding and has an R-reproductive strategy, which is in conflict with other alien crayfish species in Europe, including signal crayfish (Pacioglu et al. 2020; Galib et al. 2022). However, similar to the signal crayfish, the danube crayfish is one of the rarest crayfish species in Poland, with a rather irregular distribution. This may lead to a low overlap between local populations of the damselfly and the crayfish and, consequently, the damselfly eggs' response to this alien species may be similar to their response to IA species.

In contrast, native noble crayfish and locally invading IA spinycheek crayfish had no effect on egg survival. This suggests that the eggs from the studied damselfly populations may have evolved some resistance to the NCEs of these crayfish or that these species produce weaker CACCs that do not cause strong antipredator egg responses (Anton et al. 2020). Additionally, the ecological relevance of these predators may play a role: signal crayfish, which can strongly alter aquatic ecosystems (Nyström et al. 1996; Galib et al. 2022), may represent a higher threat to damselfly eggs than either the native noble crayfish or IA spinycheek crayfish, leading to a stronger innate response (Laverty et al. 2015).

The eggs of *I. elegans* prolonged development times under exposure to CACCs and this across all treatment groups, yet, with significant differences between native, alien and IA crayfish. Eggs exposed to IA signal CACCs showed the longest delay, whereas native noble CACCs caused the shortest delay, but still significant. This variation suggests that damselfly eggs exhibit flexible plasticity in response to predation risk and that the imposed risk is the highest under IA crayfish (Cox and Lima 2006; Sih et al. 2010). A strong delay of egg development time under signal CACCs was earlier shown in other populations of *I. elegans* (Antoł and Sniegula 2021; Amer et al. 2024), confirming that predator-induced stress responses are consistent across populations and may represent an adaptive mechanism to cope with novel predation stress. However, the opposite pattern with shorter *I. elegans* egg development under spinycheek CACCs was previously reported (Antoł and Sniegula 2021), indicating population specific responses likely associated with habitat-specific predator history (Anton et al. 2020; Mathers et al. 2022). It might be argued that prolonged egg development in the presence of cues from egg predators may carry costs. Extended egg development would indeed increase exposition time to crayfish predation (Sih and Moore 1993). This may explain why green frog (*Rana clamitans*) and East African reed frog (*Hyperolius spinigularis*) eggs hatched earlier when exposed to egg predators (Vonesh 2005; Anderson and Brown 2009). However, prolonged development times under egg predation risk may serve as a defence strategy to reduce the likelihood of hatching into high-risk larval environments (Ferrari et al. 2010). Furthermore, delayed hatching may occur as a non-adaptive result of stress-induced re-allocation of energy to costly defence mechanisms against predators (Hawlena and Schmitz 2010), away from investing in a fast embryonic development rate. It might also be that cues from generalist predators like crayfish, that are capable of preying on both egg and larval stages of the damselfly, mediate the egg response (discussed below).

The observed disruption in hatching synchrony under CACC exposure, with similar strength across all treatment groups, indicates that predator-associated cues may also affect egg cohort timing. Reduced hatching synchrony can have ecological implications, as it may reduce the effectiveness of antipredator strategies like predator satiation (Simons 2011). Studies on other *I. elegans* populations indicated that the damselfly hatching synchrony under predation stress from IA signal crayfish and native perch cues did not deviate from the control treatment (Sniegula et al. 2019; Antoł and Sniegula 2021). The discrepancy between previous and current results suggests a population specific response to predator-associated cues which might be linked to the predator history at a specific site (Anton et al. 2020).

Carry-over and cumulative NCEs on larval traits

Our study showed significant carry-over effects from the egg stage to the larval stage when *I. elegans* eggs were exposed to CACCs from native, alien and IA crayfish species. Larvae that were only exposed to CACCs during the egg stage showed reduced survival, lower body mass and reduced fat content compared to control groups, indicating that predator-induced stress effects can persist across life stages. Notably, the strength of these carry-over effects varied amongst the three crayfish types, with the most pronounced negative effects observed for the alien danube and IA signal CACCs. This pattern aligns with previous research suggesting that alien and IA predators may elicit stronger stress responses due to the absence of evolutionary exposure of prey to these predators (Cox and Lima 2006; Sih et al. 2010; Antoł and Sniegula 2021; Amer et al. 2024; Sniegula et al. 2024). The carry-over effects are consistent with the idea that stress experienced during the egg stage, including rarely documented for egg stress imposed by predation risk, can persist and manifest in later stages (Stoks and Córdoba-Aguilar 2012; Moore and Martin 2020; Sniegula et al. 2020).

Our study also indicated that larvae exposed to CACCs during both the egg and larval stages exhibited greater reductions in mass and fat content than those only exposed in the egg stage. This cumulative effect of exposure to predator-associated chemical cues indicates that the stress induced during the egg stage was not softened after hatching and that continuous exposure further intensifies the negative effects. For instance, larvae that experienced noble, danube and signal CACCs during both stages showed significantly lower mass across all treatment groups. The negative effect of predator stress on prey mass or size was earlier shown in other damselflies species and semi-aquatic insects such as mayflies (McPeek et al. 2001; Peckarsky et al. 2002). Our results add to the knowledge that continuous exposure to predator-associated cues during both egg and larval stages intensifies the physiological cost of antipredator defences.

The significant reduction in larval fat content in response to CACCs as observed in our study provides further evidence that predator-induced stress can disrupt energy allocation across life stages. In semi-aquatic invertebrates, fat reserves are critical for sustaining growth and immune function during the larval stage (Stoks et al. 2006) and their depletion due to continuous stress can impair development. In particular, the larvae exposed during both egg and larval stages to the native noble, alien danube and IA spinycheek CACCs exhibited the greatest reduction in fat content, suggesting that alien and IA crayfish impose similar physiological costs compared to native crayfish. The fact that continuous exposure to alien and IA CACCs resulted in the strongest carry-over effects suggests that invasive predators may have long-term consequences for prey populations. Further research should explore whether these carry-over effects also bridge metamorphosis and translate to reduced reproductive success in the adult stage, which could have implications for population dynamics in ecosystems invaded by alien IA crayfish.

Finally, we found no significant effect of predator-associated cues on phenoloxidase activity, our measure of investment in immune function. This happened probably because there was apparently no effect of continuous exposure and so no immediate effect, which may explain also the absence of any delayed effects. This absence of an effect on immune parameters may reflect the complex and variable nature of carry-over effects, where some traits, such as energy storage and total body mass, are more susceptible to early-life stressors than others.

Conclusions

Our study underscores the impacts of predator exposure in species with complex life cycles, where early-stage stressors can persist and negatively affect later stages. These findings are particularly relevant for predicting how non-native species, such as the IA crayfish, can alter prey populations through non-consumptive effects that accumulate over time. Given that alien and IA predators imposed stronger selective pressures on naïve prey, as found in terms of a higher effect on egg and larval survival, egg development time and larval mass and fat content, it is crucial to incorporate these carry-over and cumulative stress effects into ecological models to better predict population responses and ecosystem dynamics under biological invasions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: SS. Data curation: AA, SS. Formal analysis: SS. Funding acquisition: SS. Investigation: SS, AA, NRA, DK. Methodology: SS, RS, AA. Project administration: SS. Resources: SS. Supervision: SS. Visualization: NRA (Figure 2), SS. Writing – original draft: SS. Writing – review and editing: RS, DK, MB, NRA, AA, SS.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary data

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Data type: docx

Explanation note: An additional figure showing the effects of crayfish cues and the pond of origin on egg development time in *I. elegans*, as well as tables showing the variance explained by family (random effect) for different response traits, number of individuals per treatment group and collection pond, and multiple comparisons of proportions using Tukey's HSD contrasts.

Link: https://doi.org/10.3897/neobiota.97.139760.suppl1

Supplementary material 2

Raw data used for the statistical analysis

Authors: Szymon Sniegula, Dorota Konczarek, Maciej Bonk, Andrzej Antoł, Nermeen R. Amer, Robby Stoks

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.97.139760.suppl2