Effects of antagonistic interactions on life history and physiological traits in damselflies (Odonata, Zygoptera)

Wpływ interakcji antagonistycznych na cechy historii życiowych i fizjologicznych u ważek równoskrzydłych (Odonata, Zygoptera)

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LIST OF PUBLICATIONS

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SUMMARY

Life history strategies in organisms with complex life cycles such as insects are influenced by physiological variations and environmental factors. Yet, many of these strategies have not been explored. In my thesis, I studied how direct and indirect antagonistic interactions in combination with other ecological stressors such as seasonal time constraints and warming temperature shape growth and developmental strategies through physiological responses in two damselfly species with different life cycle characteristics. To achieve this, four separate studies were conducted:

In **Paper 1**, I investigated if non-consumptive predator effect (NCPE) of chemical cues released by a freshwater top predator, perch *Perca fluviatilis*, experienced during juvenile stages, carried over and affected life history traits at emergence in the damselfly *Ischnura elegans*. For this, a fully-crossed experimental design was used, where either or both of egg and larval stages were exposed to the predator cues. To exclude direct consumptive effects, i.e. cannibalism, larvae were reared individually.

Predator cues experienced only during the egg stage increased larval mortality rate, but only until individuals reached the second week after hatching. However, larval exposition to NCPE decreased survival rate until emergence, and individuals that survived and emerged did so with delay. Finally, larval growth rate was negatively affected by NCPE during the egg stage, and the effect increased when the exposition continued through larval stage. These results show that non-consumptive predator effects experienced only during early developmental stages, including a brief egg phase, in potential prey can carry over and negatively affect adult fitness traits.

In **Paper 2**, I studied the impact of hatching phenology and seasonal time constraints on intraspecific interactions in a cannibalistic damselfly *Lestes sponsa*, and how these variables affected cross-metamorphic life history and physiological traits. Different hatching dates commonly lead to so called size-mediated priority effects (SMPEs), where early hatched larvae take competitive advantage over late hatched ones. I hypothesized that phenologically driven SMPEs will be strengthened by time constraints imposed by the length of the growth season, the latter negatively associated with latitude. I run a fully-crossed experiment where high- or central latitude *L. sponsa* larvae were reared in groups in native and non-native combinations of temperatures and photoperiods (hereafter, thermo-photoperiods). By manipulating damselfly hatching date, and this to reinforce SMPEs, I aimed to test whether early hatchers take advantage over late hatchers in terms of life history (survival, development time, growth rate and mass at emergence) and fitness-related physiological traits (immune function measured as phenoloxidase activity, fat content and protein content at emergence).

Early hatchers had the highest survival rate, fitting SMPE prediction, yet, with no differences across thermo-photoperiods and population origin. Similarly, SMPEs was found in mass at emergence (higher mass in early than late hatched larvae), but only in central latitude *L. sponsa* reared under native thermo-photoperiod (= weak time constraint). Other life history and physiological traits did not show SMPEs. Instead, these trait expressions could be explained by different trait responses to seasonal time constraints, i.e. SMPE were outweighed by seasonal time constraints. For example, when reared under high latitude thermo-photoperiod (= strong time constraint) damselflies adaptively decreased larval development time until emergence. Strong time constraints caused decrease in immune function, indicating cost and trade-off between development rate and immune function under time constrained situation. These results imply that SMPEs are trait dependent and only weakly shaped by seasonal time constraints.

The aim of **Paper 3** was to check if and how different hatching phenology reinforcing SMPEs, in combination with altered temperatures and presence or absence of NCPEs released from perch shape life history and physiology in a cannibalistic *Ischnura elegans* sampled from a central latitude population.

Comparably to *L. sponsa* (**Paper 2**), group-reared early and late hatched *I. elegans* showed clear SMPEs in survival rate until emergence and in emergence success, with neither temperature nor NCPEs affecting priority effects in these two traits. Nonetheless, high growth temperature generated SMPE in protein content that adaptively carried over to the adult stage. Other life history traits (development time, mass and growth rate) were affected by temperature and NCPEs, but showed no support for the SMPEs. For example, predator cues reduced larval growth rate and mass at emergence, but only under high temperature treatment. This result suggests that warming temperature may magnify the effects of predator-induced stress in prey, but that the increased predation stress may not affect intraspecific SMPEs in prey. Physiological traits (immune function and fat content) were fixed across all treatments, suggesting decoupling between life history and physiology in the study population. The results confirm that antagonistic interactions reinforced by SMPEs are an important factor which, by shaping survival during the juvenile stage and emergence success, can promote early emergence of merolimnic and cannibalistic ectotherms. The results further underline that intraspecific SMPEs are only weakly affected by temperature and NCPEs.

In **Paper 4**, I verified whether different hatching dates, temperatures and corresponding changes in number of generations completed per year (voltinism) affected life history and physiological traits in high latitude populations of *I. elegans*. Here, the SMPE was not reinforced; early and late hatched groups were reared separately, hence early and later hatchers did not interact and compete for resources.

In contradiction to previous studies (**Paper II** and **Paper III**), here early and late hatchers showed no difference in survival rate, likely because of minor larval size differences within each experimental group. Minor size differences between competing organisms might result with reduced cannibalism rate, which likely happened in this experiment. Warming temperature also did not affect survival. Yet, warming shortened larval development time until emergence and increased voltinism regardless of hatching date. Early hatched individuals that experienced elevated temperature showed higher investment in immune function which might be explained by relocation of resources from other physiological traits, e.g. energy reserves. The results suggest majorly adaptive phenotypically plastic responses in life history, including voltinism, and physiological traits to climate change in organisms exposed to time constraints, and support the importance of phenological shifts in a warming world for shaping these traits in insects.

The thesis underlines the importance of antagonistic interactions in shaping fitness traits in key merolimnic ectotherms with complex life cycles. The experimental findings support the assumption that predator cues experienced during the initial developmental stage carry over to adult stage in potential prey. Such NCPEs found in the current study might ultimately have negative consequences on reproductive success and fecundity in prey. The thesis also emphasizes that within population variation in hatching date can reinforce SMPEs, and that these priority effects might have large consequences on larval and adult fitness traits, which may translate into population dynamics. Nonetheless, other environmental factors such as seasonal time constraints and warming temperature can mediate fitness-related outcomes of the SMPEs. These results highlight that the impact of antagonistic interactions on ectotherms fitness traits is largely context dependent.

STRESZCZENIE

Strategie historii życiowych u organizmów ze złożonym cyklem życia, np. u owadów, są kształtowane poprzez zróżnicowany stres fizjologiczny oraz czynniki środowiskowe. Jednak przyczyny kształtowania tak wielu strategii nadal sa słabo poznane. W mojej rozprawie badałem w jaki sposób bezpośrednie i pośrednie interakcje antagonistyczne w połączeniu z innymi stresorami ekologicznymi, takimi jak skrócona długość sezonu wegetacyjnego oraz wzrost temperatury, kształtują strategie wzrostu i rozwoju poprzez odpowiedzi fizjologiczne u dwóch gatunków ważek różniących się cechami historii życiowych. Aby to osiągnąć przeprowadziłem cztery różne badania:

W **publikacji 1** testowałem, czy niekonsumpcyjne efekty drapieżnika (NED) w postaci sygnałów chemicznych uwalnianych przez drapieżnika wyższego rzędu, okonia *Perca fluviatilis*, doświadczane przez stadia młodociane, wpływają na cechy historii życiowej *imagines* ważki *Ischnura elegans*. W tym celu użyłem krzyżowego układu eksperymentalnego, w którym stadium jaja i/lub stadium larwy były wystawione na działanie sygnałów drapieżnika. By wykluczyć wpływ bezpośrednich, tj. kanibalizmu, larwy były hodowane indywidualnie.

Sygnały drapieżnika doświadczone jedynie podczas fazy jaja zwiększyły śmiertelność larw, lecz tylko do dwóch tygodni po wykluciu. Natomiast wystawienie larw na NED zmniejszyło ich przeżywalność do wylotu, zaś osobniki, które przeżyły i wyleciały, zrobiły to z opóźnieniem. Ostatecznie NED działający na ważki znajdujące się w fazie jaja wpłynął negatywnie na tempo wzrostu larw, a efekt powiększał się, jeśli ekspozycja była kontynuowana w fazie larwalnej. Wyniki pokazują, że niekonsumpcyjne efekty drapieżnika doświadczone tylko podczas wczesnych faz rozwoju, włącznie z krótkim stadium jaja, u potencjalnej ofiary może negatywnie wpływać na cechy historii życiowych i kondycję *imagines*.

W **publikacji 2** badałem wpływ fenologii klucia i ograniczenia związanego z krótkim sezonem wzrostu na wewnątrzgatunkowe interakcje kanibalistycznej ważki *Lestes sponsa*, a także w jaki sposób opisane czynniki wpływają na cechy historii życiowych oraz fizjologii w czasie wylotu. Różne terminy klucia często prowadzą do tzw. efektów pierwszeństwa, wywołanych różnicami w rozmiarach konkurujących organizmów, z przewagą dla wcześniej klujących się osobników (EPWR). Postawiłem hipotezę, że EPWR wywołane różnicami w datach klucia będą wzmocnione dodatkowym czynnikiem – krótkim sezonem wzrostu, co uwidacznia się na wyższych szerokościach geograficznych. Przeprowadziłem krzyżowy układ eksperymentalny, w którym *L. sponsa* pochodzące z dwóch różnych szerokości geograficznych, północnej i centralnej, hodowane były w rodzimych lub obcych temperaturach i fotoperiodach (dalej termo-fotoperiodach). Celem było sprawdzenie, poprzez manipulacje dat klucia w celu wzmocnienia EPWR, czy osobniki wcześnie

klujące się będą miały przewagę nad później klującymi się w cechach historii życiowych (przeżywalność, czas rozwoju, masa przy wylocie oraz tempo wzrostu) i fizjologii, związanych z kondycją (funkcja odpornościowa mierzona aktywnością fenoloksydazy, zawartość tłuszczu i białka przy wylocie).

Zgodnie z EPWR, wcześnie klujące się osobniki wykazały najwyższą przeżywalność. Jednak różne termo-fotoperiody oraz pochodzenie geograficzne populacji nie miały wpływu na tę cechę. Podobnie EPWR stwierdziłem w masie przy wylocie (wyższa masa u larw wczesnych niż u późnych), lecz tylko u *L. sponsa* z centralnej szerokości geograficznej, które były hodowane w rodzimym termo-fotoperiodzie (= słabe ograniczenia czasowe). Inne cechy historii życiowych i fizjologii nie przejawiały EPWR, a ich ekspresje można było tłumaczyć głównie reakcjami na ograniczenia czasowe. Na przykład larwy hodowane w północnym termo-fotoperiodzie (= silne ograniczenia czasowe) plastycznie skracały czas rozwoju do wylotu. Silne ograniczenia czasowe spowodowały spadek odporności, co ujawniło koszt przyspieszonego rozwoju obniżoną funkcją odporności w sytuacji ograniczenia czasowego. Wyniki sugerują, że EPWR są słabo uwarunkowane długością sezonu wzrostu a istotność efektu pierwszeństwa zależy od mierzonych cech.

Celem **publikacji 3** było sprawdzenie, czy różne fenologie klucia wzmacniające EPWR, w połączeniu z różnymi temperaturami wzrostu oraz obecnością bądź brakiem NED, pochodzących od okonia wpływają na historie życiową i fizjologię u kanibalistycznych *Ischnura elegans*, pozyskanych z populacji w centrum zasięgu geograficznego.

Podobnie do *L. sponsa* (**publikacja 2**) grupowo hodowane wczesno i późno klujące się larwy wykazywały wyraźne EPWR we wskaźniku przeżywalności do wylotu oraz sukcesie wylotu, przy czym temperatura i NED nie wpłynęły na nasilenie efektu pierwszeństwa w tych cechach. Niemniej podwyższona temperatura wywoływała EPWR w zawartości białka w czasie wylotu. Podwyższony parametr tej cechy może mieć pozytywny wpływ na kondycję *imagines* i sukces rozrodu. Temperatura oraz NED kształtowały pozostałe cechy historii życiowych (długość rozwoju, masa i tempo wzrostu), lecz bez poparcia dla EPWR. Przykładowo, sygnał pochodzący od drapieżnika spowolnił tempo wzrostu larw oraz masę w czasie wylotu *imagines*, ale tylko w warunkach wysokiej temperatury. Wynik ten sugeruje, że podwyższona temperatura może nasilać stres u ofiary wywołany obecnością w środowisku drapieżnika, jednak nasilony stres nie wpłynie na wewnątrzgatunkowe interakcje w postaci EPWR. Co ciekawe, odporność wyrażona aktywnością fenoloksydazy i zawartością tłuszczu była stała we wszystkich grupach eksperymentalnych, co wskazuje na niezależną odpowiedź historii życiowych i fizjologii w badanej populacji. Powyższe wyniki potwierdzają, że interakcje antagonistyczne wzmocnione EPWR są ważnym czynnikiem kształtującym przeżywalności w stadiach młodocianych oraz sukces wylotu i mogą przez to

faworyzować wczesne wyloty u merolimnicznych organizmów. Natomiast dodatkowe czynniki środowiskowe jak temperatura i NED jedynie słabo wpływają na wewnątrzgatunkowe EPWR.

W **publikacji 4**, zweryfikowałem, czy różne daty klucia, temperatury oraz odpowiednie zmiany w liczbie pokoleń na rok (woltynizm) wpływają na cechy historii życiowych i fizjologii w populacji *I. elegans*, pochodzącej z północnej granicy występowania gatunku. EPWR nie zostało tutaj wzmocnione; wcześnie i późno klujące się larwy były hodowane oddzielnie, przez co wczesne i późne osobniki nie konkurowały o zasoby.

W przeciwieństwie do poprzednich wyników (**publikacja 2** i **publikacja 3**) wczesne i późne osobniki nie wykazywały różnic w przeżywalności, co mogło być wytłumaczone podobnym nasileniem kanibalizmu w obu grupach fenologicznych, spowodowanym zbyt małymi różnicami w rozmiarach larw w każdej z grup. Podwyższona temperatura również nie wpłynęła na przeżywalność. Jednak ocieplenie skróciło długość rozwoju do wylotu i zwiększyło woltynizm niezależnie od daty klucia. Wcześnie wyklute osobniki, hodowane w wysokiej temperaturze, wykazywały podwyższony wskaźnik odporności immunologicznej, co można tłumaczyć przesunięciem inwestycji zasobów z innych cech, np. tłuszczu. Wyniki pokazują znaczną plastyczność fenotypową historii życiowych i cech fizjologicznych na zmiany klimatu u organizmów wystawianych na ograniczenia czasowe oraz potwierdzają znaczenie zmian fenologicznych w ocieplającym się świecie na kształtowanie tych cech u owadów dwuśrodowiskowych o zmiennym woltynizmie.

Niniejsza praca podkreśla istotność interakcji antagonistycznych w kształtowaniu cech odpowiedzialnych za dostosowanie u kluczowych organizmów merolimnicznych o złożonych cyklach życiowych. Wyniki eksperymentów potwierdzają założenie, że stres u potencjalnej ofiary wywołany sygnałem pochodzącym od drapieżnika, który jest doświadczany jedynie w początkowych stadiach rozwoju uwidacznia się w stadium *imagines* i może ostatecznie mieć negatywny wpływ na sukces reprodukcyjny oraz płodność ofiary. Praca ta ponadto pokazuje, że zmienność dat klucia w obrębie populacji może wzmocnić efekty pierwszeństwa wywołane różnicami w rozmiarach konkurujących organizmów oraz że te efekty mogą mieć znaczny wpływ na cechy odpowiedzialne za dostosowanie, co z kolei może się przełożyć na dynamikę populacji. Inne czynniki środowiskowe, takie jak sezonowe ograniczenia czasowe i zmienność temperatur mogą dodatkowo zmieniać nasilenie efektów pierwszeństwa oraz ich konsekwencje na dobór cech. Dlatego wpływ interakcji antagonistycznych na cechy powiązane z dostosowaniem zależą w dużej mierze od kontekstu.

GLOSSARY

Abiotic factor: Non-living element of ecosystem such as temperature or precipitation interacting with other abiotic factors, as well as living (biotic) elements.

Antagonistic interaction: Direct or indirect negative influence of one organism on another organism, including predation or parasitism (Pringle, 2016).

Carry-over effect: Occurs when environmental conditions in one developmental stage, e.g. larval, influence performance and life history in a subsequence stage, e.g. adult (O'Connor et al., 2014; Wallenstein & Fisher, 1977).

Fitness traits: Also known as life-history traits, are traits which play role in organisms' reproductive success, e.g. survival until maturity, body mass and development rate (Merilä & Sheldon, 1999).

Life history: Events, e.g. hatching and maturation, and traits, e.g. body size and growth rate, measured between organism's inception to death (Roff, 2002).

Non-consumptive predator effect: Reaction of an organism to perceiving a predator presence in the environment. Such reaction may result in changes in organism behavior, life history and physiology (Hermann & Landis, 2017).

Phenology: Seasonal timing of biological events such as hatching, migration, and mating at a scale of individual organism or group or organisms (Park & Post, 2022).

Photoperiod: Length during 24 hours day cycle during which organisms are exposed to light; day length.

Priority effect: Impact of early arrival through hatching, birth or migration of one group on another group arriving later in the season. This impact can be positive, for example by improving conditions of early arrivals through facilitating access to niche space, or negative, e.g. by direct predation of early arrivals on late arrivals (Shulman et al., 1983; Weidlich et al., 2021).

Time constraint: Time limit to reach maturity and reproduce. Can be caused by seasonal decrease in temperature. Time constraint potentially causes physiological stress and leads to different compromises in organism's life history traits (Rowe & Ludwig, 1991).

Size-mediated priority effect: Priority effects caused by size difference between organisms with different arrival dates, e.g. different hatching date, with early arrivals having greater body size than late arrivals (Rasmussen et al., 2014).

Voltinism: Number of generations per year (Masaki, 1978). For example, two generations per year (bivoltine), one generation per year (univoltine) or one generation per two years (semivoltine).

INTRODUCTION

Growth rate is a key parameter in organisms life history (Nylin & Gotthard, 1998; Stearns, 1992). Growth rate shapes final body size, with the latter being often positively associated with fitness (Calsbeek & Sinervo, 2004; Honěk, 1993). Larger individuals frequently gain advantage in avoiding predator attacks (Cooper & Stankowich, 2010) and, importantly, show increased reproductive success (Byrne & Rice, 2006). One would therefore expect maximization of growth rate (Stearns, 1992). However, costs associated with fast growth, including increased predator encounter rate caused by intensified foraging (Brodin & Johansson, 2004) and physiological costs such as decreased immune function (Mangel & Stamps, 2001) are causing this trait to be optimized, and not maximized (Dmitriew, 2011). Nonetheless, a balance between benefits and costs of rapid growth strongly depend on the current environment the organism is coming across. Here, the environmental condition depends on, among other factors, antagonistic competition, availability of food resources and, in temperature regions, the degree of seasonal time constraints imposed by suboptimal temperature outside of the growth season (Laurila et al., 2001; Willi & Van Buskirk, 2022).

Recent global changes have led to increased annual temperature and greater variation in seasonal temperature patterns (IPCC, 2021). Variation in temperature plays a crucial role in shaping ectotherms' developmental traits, including growth rate, and the timing of phenological events such as dates of hatching, emergence and breeding (Parmesan, 2006). Species, populations and individuals within populations often differ in response to changes in the environmental variables that affect the mentioned phenological events (Angilletta, 2009). Individuals that arrive to the habitat early frequently show competitive advantage over late arriving ones. This competitive advantage of phenologically early organisms has been termed priority effects (Diamond, 1975; Shulman et al., 1983). The advantage is often gained through the difference in body size, with early individuals being larger than late arrivals, i.e. size-mediated priority effects, hereafter abbreviated SMPE (Geange & Stier, 2009; Rasmussen et al., 2014). In agreement with theoretical assumptions (De Meester et al., 2002) and empirical support (Boone et al., 2002; Rasmussen & Rudolf, 2015), early arrivers or early date hatchlings are likely to monopolize limited resources, escape predators, or become predators. SMPE might also cause changes in interactions at the intraspecific level such as cannibalism. Cannibalism benefits predator directly as conspecifics are rich source of energy, and indirectly through reducing competition for resources (Johansson & Crowley, 2008). Since global climate changes affect phenology at the species, population and individual level differently, knowledge about how priority effects will alter the resulting ecological interactions would itself be highly informative

and would provide valuable information for assessing future changes in this kind of interactions at each ecological level.

Antagonistic interactions can be mediated by direct consumptive and indirect non-consumptive predator effects (NCPE), the later by mere presence of chemical cues, i.e. kairomones, unintentionally produced and released to the environment by predators. It has been shown that NCPE can reduce prey fitness through risk-induced behavioral (Sievert et al., 2021), life history (Wen & Ueno, 2021) and physiological responses (Florencio et al., 2020; Katzenberger et al., 2014). For example, in order to increase its chances for survival prey may reduce its activity and foraging rate under predator's threat. Reduced activity and foraging rate decrease the chances of encountering predator, but also leads to decreased growth rate (Gehr et al., 2018; Hermann & Landis, 2017; Paterson et al., 2013; Zhang et al., 2019). Interestingly, NCPE can decrease fitness in prey to the same degree as the direct consumptive predator effect (Catalán et al., 2021; Preisser et al., 2005), hence NCPE should be considered in studies on ecological interactions, including antagonistic ones.

Variation in temperature may also affect the intensity of NCPE. Predator cues degrade faster under high temperature leading to reduced time of exposure to NCPE (Chivers et al., 2013; Gjoni et al., 2020). However, raised temperature causes ectothermic organisms to increase their activity, and this because of their increased energy needs, which might lead to higher risk of direct contact with predator (Buckley et al., 2017; Mellanby & Gardiner, 1939). Therefore, temperature and predator cues can therefore interact with each other, affecting behavioral, life history and physiological traits in prey (Gjoni et al., 2020; Truong et al., 2020).

Annual changes in temperature are particularly important for ectothermic organisms that have complex life cycles, i.e. that go through different life stages, juvenile egg and larval, and adult stage. These organisms must adjust their juvenile development and growth to seasonal changes in temperature in order to metamorphose, reach the adult stage and reproduce before the growth season ends (Rowe & Ludwig, 1991; Rudolf & Rödel, 2007). In many species, the ontogenetic changes are associated with habitat shift, for example, switch from aquatic larval stage to terrestrial adult stage (Remsburg & Turner, 2009). Also, the environmental stress might differ between the life stages and the stressor experienced in one stage might carry-over to the following stage and eventually affect fitness (Harrison et al., 2011). For instance, fecundity and reproductive investment of *Bicyclus anynana* butterfly is significantly impaired under low food availability during larval stage (Bauerfeind & Fischer, 2005). Therefore, the cross-metamorphic stage is not fully independent from the juvenile stage, and focusing on a single life stage can be misleading.

As mentioned, periodic changes in life stages, i.e. phenological events, are strongly environment-dependent, but genetic components also play a role (Wilczek et al., 2010). For example, there are insect species with fixed number of generations per year, e.g. one generation completed within a year (univoltine life cycle). Strictly univoltine species complete all ontogenetic stages within a year, regardless of the environmental background. In organisms that can complete more or less than one generation per year, i.e. that have a variable voltinism, such a switch in voltinism depends mainly on the variation in annual temperature (Altermatt, 2010; Kong et al., 2019). For example, *Popillia japonica* (Newman), a beetle mostly univoltine in its central range and semivoltine on the high latitude outskirts of its range, is predicted to increase its number of generation per year as global temperatures increase (Kistner-Thomas, 2019). Apparently, variable voltinism affects ectotherms' growth and development rate and final body size (Zeuss et al., 2017) and, as mentioned, such variation in life history traits might strongly affect antagonistic interactions.

It is worth to consider that in seasonal environments another abiotic variable, the day length or photoperiod, is an important environmental factor that cues ectotherms growth and development (MacLean, 2015; Moghadam et al., 2019; Norling, 2021). For example, in a butterfly *Lycaena phlaeas* day length affects development and growth rates, especially under lower range of growth temperatures, suggesting interactive role of temperature and photoperiod on the butterfly development (Semsar-kazerouni et al., 2022). Hence, studies on seasonal regulation of organism growth and development should consider combined effects of temperature and photoperiod, ideally across an organism life stages (Angilletta & Dunham, 2003; Lopatina et al., 2011).

To sum up, antagonistic interactions can have strong impact on fitness traits in competing organisms. The effects of these interactions can be mediated by the timing of phenological events, e.g. hatching date which might reinforce SMPEs. Other environmental factors and cues such as NCPEs, temperature and photoperiod can directly and indirectly affect rates of ectotherms growth and development. Most of previous studies investigated the effects of these factors separately, and focused on a single developmental stage, e.g. larval stage (Baranowski & Preisser, 2018; Holzmann et al., 2022; Xu et al., 2014). This thesis focuses on combined effects of aforementioned factors on damselflies' life history and physiological traits, and how these combined effects shape fitness traits during both juvenile and adult stages in the key intermediate predator in freshwater ecosystems.

OBJECTIVES OF THE THESIS

The aim of this research was to investigate how antagonistic interactions mediated by different hatching dates that reinforce size-mediated priority effects (SMPEs), non-consumptive predator effects (NCPEs), warming temperature and seasonal time constraints affect life history and physiology in damselflies - key intermediate predators in aquatic (egg and larva) and terrestrial (adult) ecosystems. In order to achieve this, the following papers were prepared:

Paper 1: The main goal of this paper was to assess whether NCPE of chemical cues released by a top predator, fish, experienced during different juvenile stages, egg and/or larval, carries-over and affect life history traits at emergence in the damselfly *Ischnura elegans*. Based on the theory and some empirical evidence, we predicted that stress caused by NCPE during juvenile stage(s) will carry-over across metamorphosis and delay emergence date, lower mass at emergence, and decrease survival until emergence in the damselfly.

Paper 2: In this article we questioned whether damselfly hatching date, and hence SMPEs in combination with different degrees of seasonal time constraints affect size-mediated priority effects in life history and physiological traits in a damselfly *Lestes sponsa*. Specifically, group-reared larvae with different hatching dates that originated from high- and low latitude populations were crossed with high- and central latitude growth conditions, i.e. temperatures and photoperiods, imposing, respectively, strong and weak seasonal time constraints. We predicted that (1) early hatched larvae will show SMPEs in life history and physiological traits over late hatched larvae. For example, early hatchers will be more cannibalistic, gain higher mass and increased immune function measured as phenoloxidase activity compared to late hatchers. (2) Strong seasonal time constraint will intensify SMPEs (environmental effect on SMPEs). And finally (3) SMPE will be greater in high latitude populations regardless of the degree of seasonal time constraints (genetic component in SMPEs).

Paper 3: The aim of this study was to assess whether and in what direction different hatching dates that reinforce SMPEs, in combination with a warming temperature and NCPE from a top predator, fish, shape in the life history and physiology in *I. elegans* from central latitude populations. We predicted that (1) early hatched larvae will show SMPEs in life history (survival until emergence, development time, mass and growth rate) and physiological traits (PO activity and protein and fat content) over late hatched larvae, whereas (2) warming will intensify and (3) NCPE will weaken SMPEs due to, respectively, increased and reduced foraging activity in damselflies.

Paper 4: In this article we studied whether different hatching dates in combination with warming temperature and associated increased number of generations per season (voltinism) affect cross-metamorphic life history and physiological traits in high latitude populations of *I. elegans*. Here, group-reared larvae with either early or late hatched individuals were crossed with current and warming temperature treatments. We predicted that (1) early hatchers will show shorter development and faster growth until emergence, higher mass at emergence, increased voltinism, and intensified antagonistic competition resulting in decreased survival rates when compared to late hatchers. (2) Warming will further decrease development time, increase growth rate and voltinism, but at the same time decrease mass and survival rates [trade-off between growth rate and body mass (or survival rate)]. (3) Early hatching and warming temperature will promote increased investment in immune function measured as phenoloxidase activity.

The hypotheses were verified during a *common-garden* experiment where I used damselflies collected in nature from central range and northern range of the species geographic distribution in Europe (Fig. 4). I field-collected *I. elegans* and *L. sponsa* adult females. These females were kept enclosed in plastic containers with wet filter paper in order to obtain eggs for the experiments. After receiving sufficient number of egg clutches, females were released into the nature at their site of origin. *Perca fluviatilis* were collected from Dobczyce reservoir in southern Poland, several weeks before experiments started. Fish were held in aquarium ($55 \times 35 \times 45$ cm), each holding 3 perch (age 1+). When the experiments ended, fish were released back into Dobczyce reservoir. Housing of fish was permitted by Local Ethical Committee (ref. 152/2018 and ref. 261/2019).

STUDY SPECIES

Ischnura elegans (Vander Linden, 1820) is a common damselfly species in Europe (Dijkstra et al., 2020), often used as a model species in evolutionary-ecological studies (Lancaster et al., 2017; Takahashi et al., 2014). It has a broad geographic distribution, ranging from Southern Spain and Cyprus to Northern Sweden. Aquatic stages (egg and larva) are usually found in standing and slow-flowing waters, whereas adult stage is terrestrial and spends large part of the time close to the freshwater habitats (Dijkstra et al., 2020). Adult females lay eggs into partially submerged and decomposed water plants. Hatching takes place 2-3 weeks after egg had been laid. Larvae molt up to 12 times and then the emergence takes place (Thompson, 1978b). The larva is the overwintering stage. Larvae and adults are predators, and show cannibalistic behavior (Müller, 1972; Thompson, 1978a). Eggs and larvae share the same water bodies with top predators, such as fish (Gall et al., 2017). Previous studies showed that this damselfly species responds to chemical cues released by higher-order predators, e.g. by reducing larval foraging activity (Gyssels & Stoks, 2006; Mikolajewski et al., 2015). The species responds to seasonal time constraints e.g. through accelerated development (Tüzün et al., 2021) and has a variable voltinism, which may lead to changes in body size (Hassall et al., 2013; Roff, 1980). Depending on the thermal conditions and latitude, *I. elegans* can complete one (univoltine) or two generation within a year (bivoltine) in central Europe and one generation within one year or one generation within two years (semivoltine) in northern Europe (Corbet et al., 2006, Ulf Norling pers. comm.). Because phenological events such as emergence, breeding and egg laying are asynchronous in time, different larval size cohorts share the same habitat (Corbet, 1999; Thompson, 1978b). Occurrence of different size-cohorts creates opportunity for intraspecific size-mediated priority effects (Anholt, 1994; Hopper et al., 1996; Rasmussen et al., 2014).

Figure 2. Larva (left) and adult female (right) of *Ischnura elegans*. Pictures taken by Ch.J. Sharp (larva) and L.B. Tettenborn (adult) and shared under Creative Commons Attribution-Share Alike 3.0 Unported license (creativecommons.org/licenses/by-sa/3.0/).

Lestes sponsa (Hansemann, 1823) is a damselfly species with wide Eurasian distribution. In Europe, In Europe it ranges from Central Spaind and Eastern Turkey to Northern Sweden (Dijkstra et al., 2020). They prefer still, fresh water, both permament and temporary, with abundant vegetation (Dijkstra et al., 2020). Juvenile stages are aquatic (although eggs are often laid above water) while the adult stage is terrestrial. Larvae and adults are predators, including cannibals. *L. sponsa* is strictly univoltine (Corbet, 1999; Dańko et al., 2017; Norling, 2018). The species overwinters in the egg stage, and eggs enter winter diapause 2-3 weeks after being laid (Corbet, 1999). Hatching takes place during the following spring and the length of larval development takes a couple of months, and depends on water temperature and photoperiod (Corbet, 1956; Śniegula & Johansson, 2010). Emergence occurs usually after 10 moults (Pickup & Thompson, 1990). The damselfly is responsive to time constraints; larvae increase their growth and development under long photoperiod which indicates advancement of the growth season and/or high latitude conditions (Johansson et al., 2021). *L. sponsa* increases cannibalistic behavior with the degree of seasonal time constraints (Pickup et al., 1984; Sniegula et al., 2017). Larvae share habitats with fish as top predators, and presence of fish can impact damselfly's foraging behavior and physiological and life history traits (Johansson et al., 2001; Stoks, 2001).

Figure 3. Larva (left) and adult female (right) of *Lestes sponsa*. Pictures taken by M. Barlow (larva) and Ch.J. Sharp (adult) and and shared under Attribution-NonCommercial 2.0 Generic (CC BY-NC 2.0) (creativecommons.org/licenses/by-nc/2.0/ picture left) and Creative Commons Attribution-Share Alike 3.0 Unported license (creativecommons.org/licenses/by-sa/3.0/ picture right)

Perca fluviatilis L. is a predatory and insectivorous fish species commonly used as a model organism in predator-prey study systems (Henderson et al., 2017; Hirsch & Eckmann, 2015). It is widely occurring, opportunistic fish living in lakes, streams, and brackish waters. The fish live up to 21 years, although usually up to 6 years (Kottelat & Freyhof, 2007). Its generation time takes about 3 years, with reproduction occurring once per year (Craig, 2000). As most other predatory species, perch synthesizes and releases to the environment chemical substances, kairomones, perceived by prey as predatory cues (Kasumyan, 2022; Van Gool & Ringelberg, 2002).

Figure 4. *Perca fluviatilis* as one of the top predators in freshwater ecosystems. Cropped picture, original taken by G.S. Martin and shared under Attribution-ShareAlike 2.0 Generic (CC BY-SA 2.0) license (creativecommons.org/licenses/by-sa/2.0/deed.en)
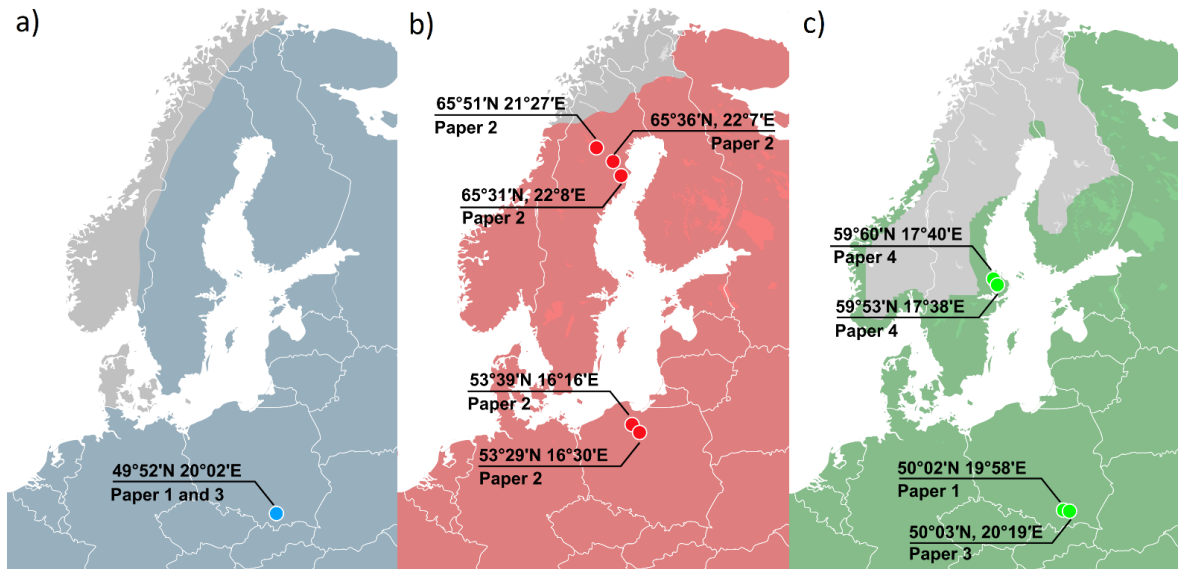
Figure 4. Collection sites for a) *Perca fluviatilis* b) *Lestes sponsa* c) *Ischnura elegans* used during the experiments. Colored areas represents geographical ranges of respective species (modified from Dijkstra et al., 2020; Stepien & Haponski, 2015). Cropped and edited picture, original made by Nordwestern and shared under Creative Commons Attribution-Share Alike 4.0 International (creativecommons.org/licenses/by-sa/4.0/deed.en)

CONCLUSIONS

- Stress caused by mere presence of chemical cues from a predator experienced during juvenile *Ischnura elegans* stages carried over to the damselfly adult stage. Specifically, larval survival and growth rate were negatively affected by chemical cues experienced only during a brief egg stage, and these effects were carried over across metamorphosis, supporting the hypothesis that NPEs considerable impact fitness traits in the consecutive developmental stage(s) in organisms with complex life cycles.

- Co-occurrence of early and late hatched damselflies caused elevated survival of the former, and a drop in survival of the latter. The hatching phenology effect on survival disappeared when early and later hatchers did not share the same environment, confirming presence of SMPE mediated by hatching phenology. SMPE were also found in mass at emergence in central latitude *Lestes sponsa* reared under weak time constraints conditions, and protein content at emergence under increased temperature conditions in *I. elegans*. No SMPE occurred in other life history traits, indicating that these traits were shaped principally by environmental factors such as seasonal time constraints, temperature and NPEs.

- Independently of SMPE in survival rate, high latitude *L. sponsa* reacted to strong seasonal time constraints by increasing development rates, but this with a cost of decreased immune function, indicating a trade-off between these two traits under time constraint situation. Central latitude *I. elegans* decreased growth rate and body mass at emergence in response to predator cues, but only under increased temperature, suggesting a trade-off between increased survival under predation pressure and decreased growth rate under warming conditions. This implies that warming temperature can have amplifying effects of predator stress during the aquatic larval stage, resulting in reduced measures of fitness traits such adult mass. These costs and trade-offs in life history traits were not reflected in physiological traits, suggesting a decoupling between life history and physiology in the studied population of *I. elegans*.

- Increased temperature accelerates larval development, which is in line with other studies on ectotherms. In case of *I. elegans* from high latitude populations, warming temperature shortened larval development time and increased voltinism, regardless of hatching

phenology. However, early hatched individuals grown under increased temperature had elevated immune function. One possible explanation is relocation of energy resources and higher investment into traits responsible for defense against pathogens which multiply faster under increased temperature conditions. Aforementioned results strongly suggest adaptive responses of high latitude, strongly time constrained populations to warming.

- A key finding in this thesis was that, although early or advanced in time hatched damselflies are better able to monopolize limited resources over late hatched individuals, this conclusion can be generalized only to some extent since the magnitude of SMPE enforced by hatching phenology is species and population dependent, and can be further mediated by environmental factors as such NCPE, temperature and seasonal time constraints. Hence, SMPE is context dependent. Future studies should focus on intra- and interspecific interactions in other taxa and investigate mechanisms underlying population-specific outcomes of these interactions in the context of anthropogenic factors.

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PAPER 1

Effects of predator cues carry over from egg and larval stage to adult life-history traits in a damselfly

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Effects of predator cues carry over from egg and larval stage to adult life-history traits in a damselfly

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Abstract: Non-consumptive predator effects experienced in early life stages of prey may result in life-history costs in later life stages. Such effects can, for example, alter the growth rate during the juvenile stage, which may carry over to size at maturity. However, we have limited knowledge of the carry-over effects starting from the egg stage through the larval stage to the adult stage. Here, we present results from a laboratory experiment in which we exposed a damselfly, *Ischnura elegans*, to chemical cues originating from a fish predator, perch. We used a 2×2-full-factorial design in which the damselflies were exposed to predator cues during either the immobile egg or the mobile larval stage. The presence of predator cues, i.e., non-consumptive predator effects, during the egg stage caused decreased survival, but only until 2 wk after larval hatching. Predator cues during the larval stage caused decreased survival until emergence and an increase in development time until emergence. However, mass at emergence was not affected by predator cues. When fish cues were present in the egg or larval stage, there was a lower growth rate in final-instar larvae than in larvae that did not receive fish cues. Our results add to the growing number of studies showing that predation-risk cues in the egg stage can carry over to the adult stage, which ultimately could have consequences for adult life-history traits, such as survival and fecundity.

Key words: predator cue, carry-over effects, life history, kairomone, predator–prey interaction, phenotypic plasticity, complex life cycle

Indirect non-consumptive effects of predators on prey are defined as effects that do not kill the prey but incur costs, such as reduced feeding, a change in diet, or increased physiological stress (Peacor and Werner 2000, Stoks et al. 2005, Hawlena and Perez-Mellado 2009). Direct consumptive effects are defined as the killing of the prey. Indirect non-consumptive effects and direct consumptive effects are also referred to as trait-mediated and density-mediated effects, respectively (Abrams 2007).The costs of indirect non-consumptive effects usually have a negative effect on survival and reproduction (Zanette et al. 2003, Creel et al. 2009) and can, thus, have important consequences for population and community dynamics (Abrams 1995, Preisser et al. 2005). The impact of indirect non-consumptive effects can be as strong as or stronger than that of direct consumptive effects (Preisser et al. 2005). Information on how indirect non-consumptive processes affect survival, development, and size at maturation can, thus, provide an important mechanistic understanding of how these impacts may affect population dynamics.

Many organisms have complex life cycles, defined as ontogenetic changes in physiology, morphology, or behavior associated with a habitat shift (Wilbur 1980). For example, many aquatic insects start as eggs, following which they undergo an aquatic larval stage and then shift to a terrestrial stage during which maturation and mating occur. It is reasonable to assume that predation risk may differ among these 3 stages: egg, larva, and adult (Wilbur 1980). It is, therefore, interesting to examine whether the effects of exposure to non-consumptive predation risk carry over through these stages and to compare the strength of these effects between stages. Such studies are relevant because predation risk may vary across space and time during an organism's ontogeny (Tolon et al. 2009).

Past studies have found strong carry-over effects between the larval stage and the adult stage in amphibians and insects. When exposed to predation cues (i.e., non-consumptive predation risk), these organisms usually show longer development times and a larger size at metamorphosis, although other patterns might occur (Ball and Baker 1996, Laurila et al. 1998, Benard 2004, Costanzo et al. 2011, Sniegula et al. 2019b). However, few studies have focused on how predation

risk during the egg stage affects the later larval and adult stages and whether a switch from high-predation risk to low-predation risk over ontogeny can be compensated for in later ontogenetic stages. Nevertheless, prey can detect predator cues in the egg stage and show earlier or later hatching in response to predation risk during this stage (Warkentin 1995, 2011, Moore et al. 1996, Sniegula et al. 2019a).

Since eggs respond to non-lethal predation cues, it is interesting to examine how such cues affect later life-history stages. In one of the few studies that have explored how predation risk in the egg stage affects adult traits, Fontana-Bria et al. (2017) found that non-lethal predation cues from predatory damselfly larvae affected wing size but not adult size in a damselfly. They also found that the same cues caused longer development times until metamorphosis in females but not in males of a mosquito (Fontana-Bria et al. 2017). However, more studies are needed to explore the commonness of these carry-over effects from the egg stage into the adult stage.

Here, we examine how non-lethal predator cues experienced during the egg stage, larval stage, or both affect the survival, development, growth of larvae, and mass at emergence of the damselfly *Ischnura elegans* (Vander Linden, 1820). We predicted that eggs or larvae exposed to predator fish cues would have lower survival until emergence, lower mass at emergence, and a longer development time until emergence in comparison to controls that did not experience such cues in the egg or larval stage. We based our predictions on the flexible growth and development time effort model in Abrams and Rowe (1996). However, many more scenarios for these life-history traits are possible (Abrams and Rowe 1996, Benard 2004).

METHODS

Experimental design

We used a laboratory experiment with a complete 2×2 design in which damselfly (*I. elegans*) eggs and larvae were sequentially exposed to fish cues or no fish cues (Fig. 1) to examine

indirect non-consumptive effects of predator cues and assess how these effects influenced life-history traits in larval and adult stages.

We caught adult *I. elegans* females at Płaszów Pond, Poland (50°02'26"N, 19°58'15"E) on 20 July 2018 using standard methods (Sniegula et al. 2019a). The species has an aquatic egg and larval stage followed by a terrestrial adult stage. Eggs and larvae commonly share habitat with fishes (Corbet 1999, Le Gall et al. 2017, Sniegula et al. 2019a). Fishes present at Płaszów Pond include European Perch (*Perca fluviatilis* Linnaeus, 1758). We captured 15 female damselflies and placed them in individual plastic cups with wet filter paper for egg laying. Perforated lids that allow ventilation were put on the cups. Immediately after collection, we transported cups with females by car to the Institute of Nature Conservation Polish Academy of Sciences, Kraków, Poland.

Upon arrival to the laboratory, we placed the cups with females in a room with natural light conditions at room temperature. Ten females laid eggs within the next 2 d. Thereafter, we released all females into the wild. Each clutch (family) contained hundreds of eggs, and we used 10 families for the experiment. We split each family into 2 halves, which we placed separately in plastic containers ($15 \times 10 \times 6.5$ cm) filled with 400 mL of dechlorinated aged tap water. We treated ½ of each clutch with perch cues as described below. We designated the other ½ as a control, which received no perch cues. On the day of hatching (8 August 2018), we divided the perch treatment group into 2 subgroups: the perch (egg)–perch (larval) treatment subgroup and the perch (egg)–no perch (larval) treatment subgroup. We also divided the control group into 2 subgroups: the no perch (egg)–perch (larval) subgroup and the no perch (egg)–no perch (larval) treatment subgroup (Fig. 1).

At hatching, each treatment received 5 larvae from each family, i.e., 20 larvae/family were included in the experiment. Each treatment had 10 replicates (families); thus, there were 200 individuals at the start of the experiment. We randomly chose 5 individuals from each family and moved them to individual 200-mL plastic cups filled with 100 mL of aged tap water. The larvae shared the same cups for another 15 d to increase survival (De Block and Stoks 2003). Next, we placed the larvae individually in 200-mL plastic cups and treated ½ with perch cues and ½ without

perch cues, the same way as during the first 15 d after hatching. Throughout the experiment, we fed larvae twice/d with nauplii of *Artemia salina* (mean = 201.8 nauplii/portion, standard error = 6.71 nauplii, n = 10 portions). After larvae entered the pre-final instar, we supplemented their diet with 3 standard-size *Chironomidae* larvae every Monday, Wednesday, and Friday.

Predator-cue treatments

To provide constant predatory cues for the perch treatment groups (Van Buskirk et al. 2014, Sniegula et al. 2019a), every other day we replaced ¼ of the water in the experimental containers holding damselfly eggs and larvae with water from an aquarium (55 × 35 × 45 cm) holding 3 perch (age 1+ y). In the control group, we replaced the same amount of water with aged tap water (Fig. 1). We captured perch from Dobczyce Lake (49°52'27"N, 20°2'55"E) several weeks before the experiment started and housed them with permission from the Local Ethical Committee (ref. 152/2018). We fed perch once/d with frozen *Chironomidae* larvae.

Response variables

We measured the following life-history variables: larval survival, development time, mass up to and at emergence, growth rate to final instar, and mass of the final instar as a function of emergence success. Larval survival was measured as the number of larvae alive after 2 wk and when entering the final instar. Survival until emergence was measured as the number of larvae that emerged successfully. Development time was measured as the number of days between hatching and emergence, and mass at emergence was measured as wet mass. The mass of the final larval instar and adult at emergence was measured as the wet mass of the larvae entering the final instar and as the wet mass of adult the day after emergence. Prior to weighing, we dried the larvae by blotting them with tissue paper. The growth rate to the final larval instar was measured as the mass of larvae entering the final instar/number of days between hatching and entrance into the final instar. We chose to measure mass and growth rate during the final instar because these values

provided an additional mechanistic explanation for the life-history traits we measured at emergence.

We used mass at emergence rather than adult structural size (measured, for example, as head width) as our measure of adult size. We note, however, that these 2 variables are used as surrogates for predicting insect fitness (Sokolovska et al. 2000, Stillwell et al. 2010). In addition, damselfly head widths of the final instar are correlated with adult head widths, and head widths are correlated with overall structural body size (Harvey and Corbet 1985, Corbet 1999). We used AS.62. R2 Plus Analytical Balance (Radwag®, North Miami Beach, Florida) balance for larval and adult weighing.

Statistical methods

To measure the survival rate, we used a generalized mixed model with a binomial distribution using the glmer function in the *lme4* package in R (version 6.3.1, R Project for Statistical Computing, Vienna, Austria; Bates et al. 2015). We used Wald χ^2 statistics to test for the fixed effects of 2 categorical explanatory variables: the presence/absence of fish cues in the egg and larval stages, and their interaction (Fox and Weisberg 2019). In case of substantial interaction terms, we assessed the differences between treatment levels using the emmeans function from the *emmeans* package in R (Lenth et al. 2020). To test for those same fixed effects of presence/absence of fish cues on development time (days), wet mass, and growth rate, we used linear mixed models implemented in the *lme4* package with the lmer function (Bates et al. 2015). To measure whether there was a minimal mass the larvae had to reach to emerge successfully, we used a logistic mixed-effects model with a binomial distribution (glmer function) to test emergence success as a function of final-instar wet mass. We used family as a random effect in all models.

RESULTS

Survival

2 wk Survival of larvae for the first 2 wk after hatching was negatively affected by fish cues presented in the egg stage, but only when fish cues were also present in the larval stage (Fig. 2A,

Tables 1, S1). Survival was reduced by 43% when only the larval stage was exposed to fish cues but was reduced by 75% when both the egg and larval stages were exposed to fish cues (Fig. 2A, Table S1). This result suggests an interaction between egg and larval fish-cue treatments, but the interaction had a negligible effect in our model (Table 1).

Final instar before emergence   Survival to the final instar was 50% lower in treatments where fish cues were present during the larval stage. There was no substantial effect of fish cues received in the egg stage (Fig. 2B, Table 1). Hence, as larvae developed and grew, the effect of fish cues received in the egg stage diminished.

Emergence   Survival to emergence was marginally affected by fish cues received in the larval stage (Table 1): larvae receiving fish cues showed lower survival than those that did not (Fig. 2C).

Development time and mass at emergence

There was a substantial overall effect of fish cues received in the larval stage on development time until emergence (Fig. 3A, Table 1). Larvae that received fish cues only in the larval stage took on average 6 d longer to develop than those that did not, and there was a similar difference in development time when fish cues were presented in both the egg and larval stages (Fig. 3A). In contrast, development time was not affected by fish cues received in the egg stage only, and there was no egg by larval fish cue interaction effect (Table 1).

There was no substantial effect of the treatments on wet mass at emergence (Fig. 3B, Table 1), suggesting that fish cues received during either or both egg and larval stages do not affect mass at emergence.

Growth rate

Growth rate based on mass when larvae entered the final instar was much lower in treatments where fish cues were present in either the egg or larval stage or both (Fig. 4, Table 1). For larvae that received no fish cues in the larval stage, growth rate was reduced by 13% when fish cues were presented in the egg stage. However, growth rate was reduced by a further 8% when fish cues were present in both the egg and larval stages (Fig. 4, Table S2). Hence, the effect of fish cues was stronger when they were received in both the egg and larval stages than when they were received in the egg stage only (Fig. 4). This finding suggests an interaction between egg and larval fish-cue treatments, but our analyses indicated the interaction was only marginal (Table 1).

Emergence success by mass of final instar

Wet mass at the final instar had a positive effect on larval emergence success ($df = 1$, $\chi^2 = 6.63$, $p = 0.01$; Fig. 5).

DISCUSSION

In this study we examined how the non-consumptive presence of a predator during the egg stage, larval stage, or both affect the life-history traits of the damselfly *I. elegans*. We found that indirect non-consumptive effects of fish cues decreased survival to emergence and the development time to emergence in the damselfly. These results correspond with those from previous studies (Tseng 2003, Hellmann et al. 2011, McCauley et al. 2011, Meadows et al. 2017) and, thus, further confirm that indirect non-consumptive effects are present in many animals with complex life cycles (Benard 2004). The novelty of our experiment was that we looked across 3 developmental stages: eggs, larvae, and adults. However, effects of the fish-cue treatments during the egg stage were not strong for any response variable measured in the larval and adult stages, except larval growth rate to the final instar.

Survival until emergence was substantially affected by the presence of fish cues in the larval stage. Such an effect on survival until metamorphosis has also been found in other studies. For example, Baranowski and Preisser (2018) found that the non-consumptive exposure of luna moth larvae to predatory wasps resulted in increased luna moth mortality, McCauley et al. (2011) showed a strong non-consumptive lethal effect of fishes or predatory insects on dragonfly larvae, and Ower and Juliano (2019) showed that presence of chemical cues of the predacious elephant mosquito decreased eastern tree hole mosquito survivorship until emergence. Short-term behavioral and life-history studies often show that organisms are affected by fish cues (Silberbush et al. 2019, Sniegula et al. 2019a), but other studies have shown that the effects on behavior and growth decline over the long term (Dalesman et al. 2015). Here, we showed that the effects of fish cues experienced during either or both egg and larval stages persisted until emergence because survival up to emergence was reduced, suggesting that the non-consumptive effects come with high costs in terms of mortality.

Development time was substantially longer for damselflies receiving fish cues in the larval stage than those that did not. This longer development time was likely responsible for the lack of an effect of fish cues on mass at emergence. Hence, larvae that experienced stressful fish cues compensated for their slower growth by developing for a longer period of time. Similar results have been found in other damselflies (Johansson et al. 2001, Mikolajewski et al. 2005) as well as in other animals (Ball and Baker 1996, Wildy et al. 1999). Interestingly, Stoks et al. (2012) observed the opposite response in larval damselfly growth rate in the presence of invertebrate predator cues. These contrasting results could be caused by the functional difference in predators used in the studies. The slow development and growth we observed might be adaptive because the predator we used is not gape-limited (McCoy et al. 2011). In contrast, in the Stoks et al. (2012) study, the invertebrate predator used, a dragonfly larva, was gape-limited. Because damselfly larvae cannot reach a size refuge from predation by perch, they probably reduce their foraging activity to reduce predation risk during the larval stage. A lower activity results in a lower encounter rate with food,

but also a lower encounter rate with predators (Leonardsson and Johansson 1997). The former reduces growth and development and the latter reduces mortality risk. Reduced foraging activity in the presence of non-gape-limited predator cues is often found in insect larvae (Johansson et al. 2001, Davenport et al. 2014). In contrast, if the predator is gape-limited, potential prey might be able to grow to a sufficient size to reach a safe size refuge from predation (Urban 2008). In the absence of large top predators, such as fishes, individuals investing in rapid growth to reach a large size within a short time might have an advantage in terms of survival (Urban 2008). Nevertheless, larval development time is important for the adult stage because studies have shown that mating success is affected by the adult life span (Cordero et al. 1997, Thompson et al. 2011, Tuzun and Stoks 2018), and those individuals that emerge earlier during the favorable season are expected to have a longer life span (Rowe and Ludwig 1991).

We found that growth rate to the final instar was negatively affected by predation cues in the egg stage, although there were no other strong effects of fish cues being present only in the egg stage. In a previous study, we found that the stress imposed by the presence of fish cues during the egg stage increased egg mortality by ~60% in *I. elegans* (Sniegula et al. 2019a), suggesting that predation-risk stress in the egg stage can be high. Nevertheless, for those eggs that survived, we found that the larvae were able to compensate for this egg-stress effect, since they showed no differences in development time compared to the control larvae. Such compensation in life-history traits also occurs in other invertebrates (Dalesman et al. 2015, Fontana-Bria et al. 2017). Nonetheless, individuals exposed to fish cues during the short-lasting egg stage did not compensate for it in terms of increased larval growth rate. Rather, fish cues received during the egg and larval stages likely caused physiological stress or behavioral modifications that reduced growth (Johansson et al. 2001), and slower growth resulted in low emergence success and, thus, higher mortality at emergence. In fact, those individuals that emerged had a higher mass at final instar before emergence compared to those that did not emerge, indicating the existence of a threshold mass the larvae must reach to successfully emerge. However, compensation may come with costs.

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For example, individuals exposed to predation cues during the egg stage developed smaller wings than individuals not experiencing these cues (Fontana-Bria et al. 2017). Wing size commonly affects flight performance in insects, including damselflies (DeVries et al. 2010, Sacchi and Hardersen 2013). Interestingly, exposure to predation risk in the egg stage in the Ringed Salamander, *Ambystoma annulatum* Cope, 1886, resulted in reduced activity in larvae (Mathis et al. 2008), suggesting that life-history traits are not the only traits affected by exposure to predation risk in the egg stage.

In summary, we show that non-consumptive predation stress during the egg or larval stage can have negative effects on key life-history traits through ontogeny. Thus, we showed carry-over effects of predator cues across the developmental stages of prey, indicating the importance of considering predation effects starting from the initial egg stage. Future experiments should focus on predation stress imposed separately and in concert at different life stages, as exposure to predator cues during different life stages may have different effects on the life history of prey species throughout development during their complex life cycles. Such effects may be important under natural conditions, since predation risk often varies over space and time during an organism's ontogeny (Tolon et al. 2009).

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Fig. 1. The full factorial experimental design. Initially, damselfly (*Ischnura elegans*) eggs were assigned to groups receiving either fish cues or no fish cues. On the hatching day, each group was divided into 2 subgroups, resulting in a full factorial design in which the 2 predator cue conditions in the egg stage were fully crossed with 2 predator cue conditions in the larval stage, resulting in 4 treatment combinations.

Fig. 2. Proportion of *Ischnura elegans* larvae surviving 2 wk after hatching (A), until entrance into the final larval instar before emergence (B), and to emergence (C). Error bars are ± 1 standard error.

Fig. 3. Development time of larval *Ischnura elegans* until emergence (A) and wet mass at emergence (B). Error bars represent ± 1 standard error.

Fig. 4. Growth rate of larval *Ischnura elegans* to the final larval instar based on wet mass. Error bars represent ± 1 standard error.

Fig. 5. Emergence success as a function of the wet mass of the final larval instar of *Ischnura elegans*.

Table 1. Results from mixed-model analysis examining the effect of fish cues received in the egg and larval stages on life-history traits in *Ischnura elegans.* Each treatment combination (i.e., 2 predator cue conditions in the egg stage fully crossed with 2 predator cue conditions in the larval stage) was replicated 10× for a total of 40 replicates.

Predictor	df	χ^2	*p*-value
Survival 2 wk after larval hatching			
Egg	1	3.720	**0.05**
Larva	1	23.305	**<0.001**
Egg × larva	1	3.313	0.069
Survival until entrance into final instar			
Egg	1	0.389	0.533
Larva	1	14.525	**<0.001**
Egg × larva	1	0.794	0.373
Survival until emergence			
Egg	1	1.134	0.287
Larva	1	3.799	**0.051**
Egg × larva	1	1.647	0.199
Development time until emergence			
Egg	1	0.802	0.371
Larva	1	12.875	**<0.001**
Egg × larva	1	1.21	0.271
Mass at emergence			
Egg	1	0.057	0.811
Larva	1	2.24	0.135
Egg × larva	1	0.003	0.953
Growth rate until final instar (mass)			
Egg	1	6.041	**0.014**
Larva	1	15.288	**<0.001**
Egg × larva	1	3.148	0.076
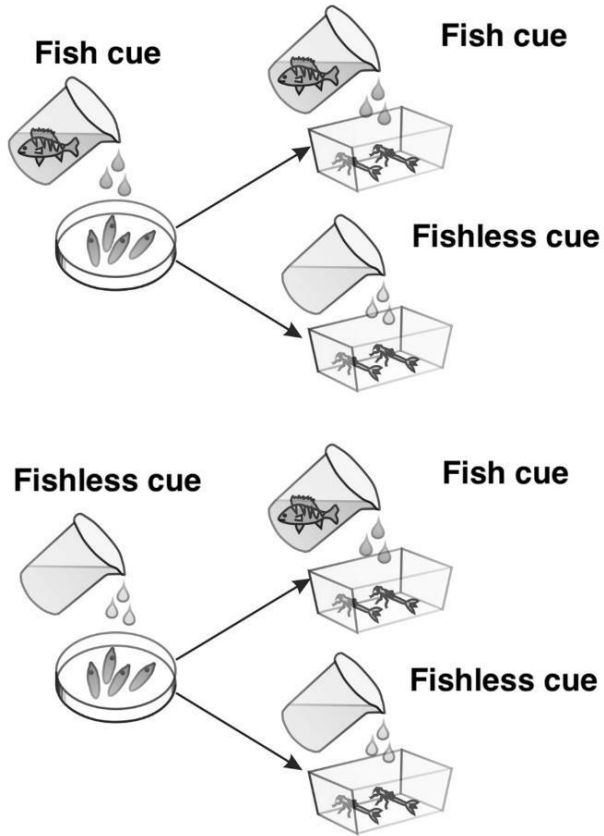
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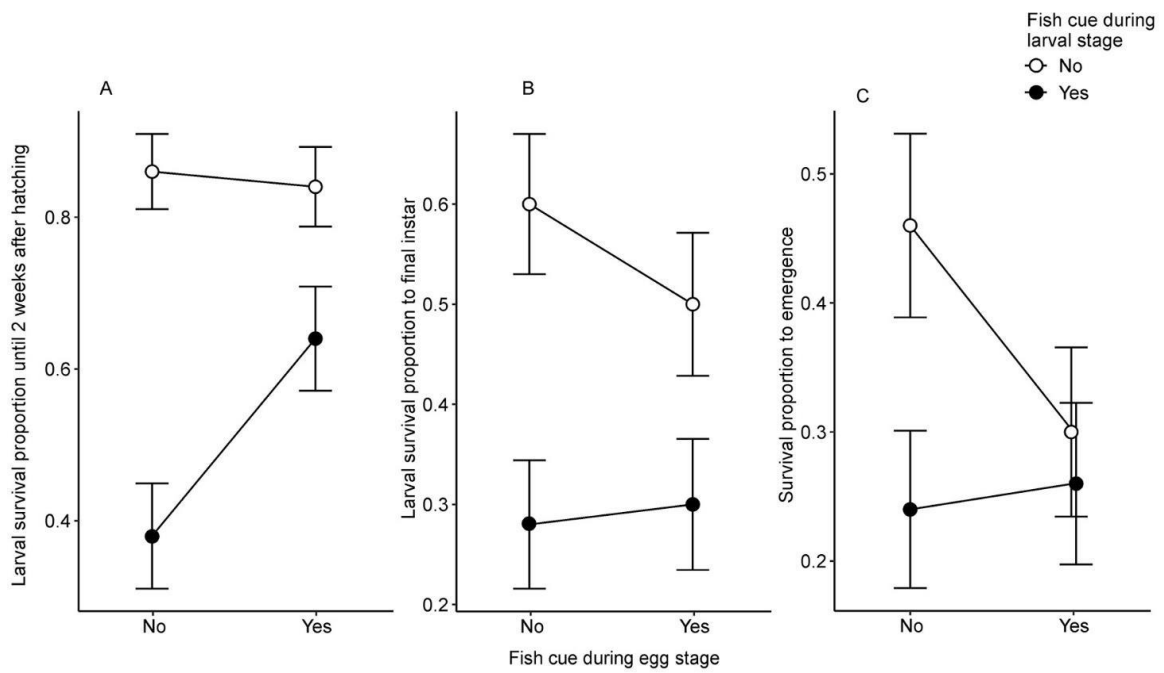
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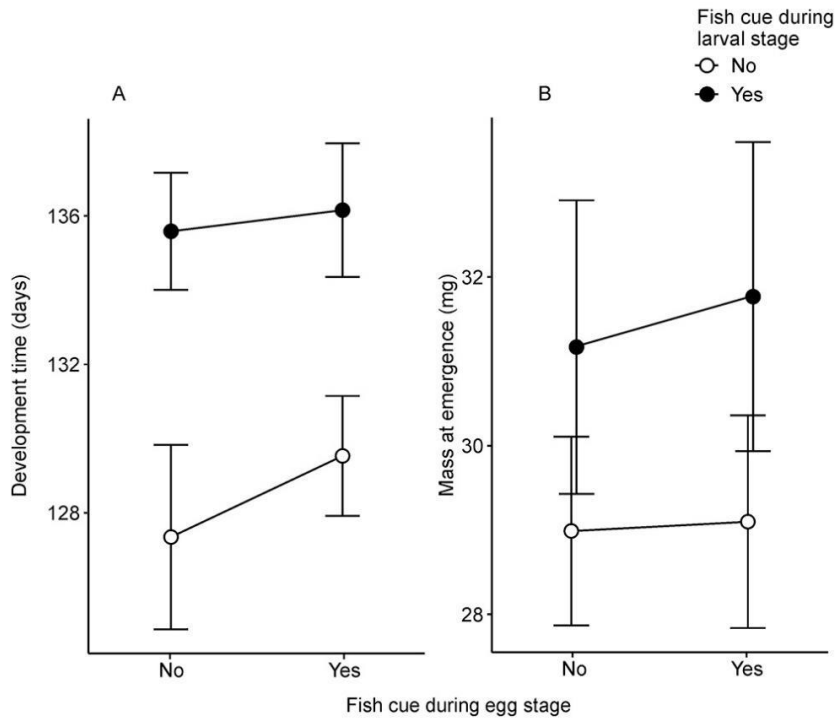
Fig. 3.
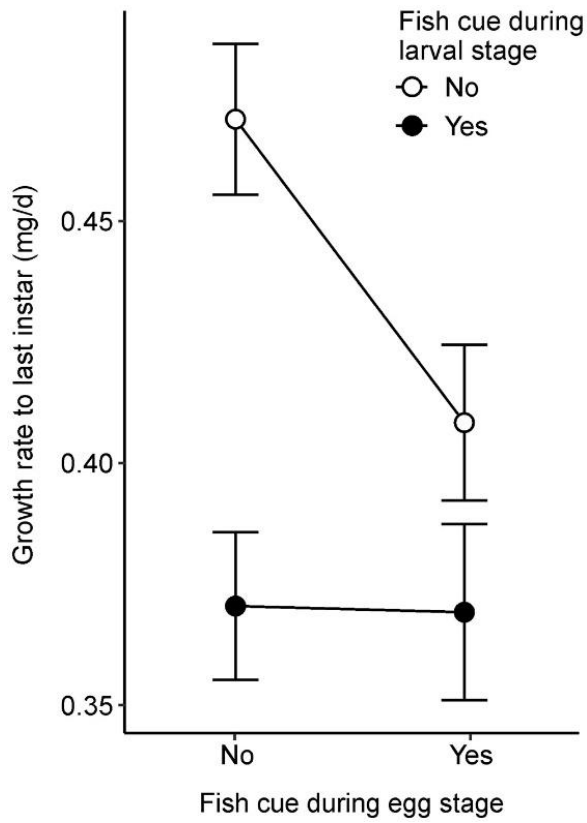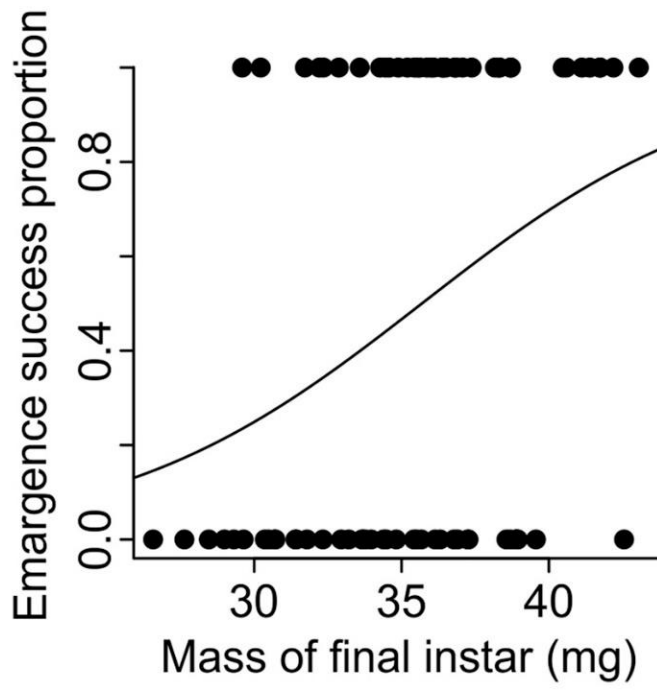

Fig. 4.

Fig. 5.

PAPER 2

Size-mediated priority effects are trait-dependent and consistent across latitudes in a damselfly

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OIKOS

Research

Size-mediated priority effects are trait-dependent and consistent across latitudes in a damselfly

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Variation in hatching time (phenology) might cause size differences within populations resulting in size-mediated priority effects (SMPEs) shaping intraspecific interactions. These phenology-driven effects potentially can be strengthened by seasonal time constraints caused by a short growth season, and depend on latitude. Here the single and combined effects of phenology and latitude-associated time constraints on SMPEs in larvae of an aquatic insect, the damselfly *Lestes sponsa*, are studied. We did so by rearing larvae in groups of 16 individuals with different phenology (hatching date) thereby imposing strong intraspecific competition, resulting in cannibalism. We thereby manipulated in a fully crossed way time constraints (combination of temperature and photoperiod: thermo-photoperiod) in larvae from low-latitude and more time constrained high-latitude populations, and examined effects on life history (survival, development, growth) and physiology (fat and protein contents, and phenoloxidase activity as a measure of immune function). Phenology, time constraints and latitude of origin had strong effects on life history, but only the time constraint affected the physiology. We detected a SMPE for survival that, however, was not stronger under time constraints and was consistent in strength between latitudes. Phenology and time constraints interacted for development and growth in a direction suggesting adaptive responses to time constraints but these life history traits did not show SMPEs. We provided important insights in the study of SMPEs thereby showing these to be trait-dependent and not more pronounced under experimentally manipulated or latitude-associated time constraints. Our study thereby makes an important addition to geographic variation in SMPEs, a largely neglected topic.

Keywords: cannibalism, competition, life history, physiology, priority effect, time constraint

Introduction

Year-to-year variation in seasonal temperature patterns affects arrival to the breeding sites, hatching and other phenological events that might alter antagonistic interactions such as competition and predation (Rasmussen et al. 2014, Bailey and van de Pol 2016).

Antagonistic interactions are indeed expected to change since species, populations and individuals show genetic and phenotypic variation in phenological events (Yang and Rudolf 2010, Sniegula et al. 2016c). Hence, not all species, populations and individuals within populations react in the same way to environmental changes. These temporal changes in phenological events may lead to changes in priority effects, where advanced organisms may have a competitive advantage over non-advanced ones often through a size-mediated benefit (Yang and Rudolf 2010). Advanced individuals in terms of arrival or hatching are expected to monopolize limited resources, escape predators or become predators of non-advanced individuals (Rasmussen et al. 2014). Size-mediated priority effects (SMPE) (Rasmussen et al. 2014) are likely widespread as these have been recorded in several taxa (e.g. salamanders: Eitam et al. 2005; dragonflies: Rasmussen et al. 2014; damselflies: Sniegula et al. 2019).

While it is well-known that antagonistic interactions may show geographic variation (Urban 2008, Sniegula et al. 2017a), this has largely been neglected for size-mediated priority effects (Young et al. 2015). Along latitudinal gradients changes in the strength of SMPE can be expected because two key environmental conditions that differ across latitudes may shape their strength. First, seasonal time constraints imposed on ectothermic organisms, especially those living in time-limited high-latitude conditions, can alter the strength of SMPE. In agreement with life history theory (Abrams et al. 1996), empirical studies indicate that organisms facing seasonal time constraints show compensatory responses in terms of a shorter juvenile development and/or faster growth in order to reach a minimal size at emergence, and to mature and breed before the end of a brief growth season (Johansson and Rowe 1999, Dmitriew 2011, Salminen et al. 2012). These adjustments are commonly triggered by environmental cues, mainly day length or photoperiod (Nylin and Gotthard 1998). Time constraints might lead to increased SMPE, because individuals that are late in the season or experience a short growth season become more active in searching for food, which in turn might affect risk taking under predation pressure (Stoks et al. 2005, 2006a, Sniegula et al. 2017a). Despite the expectation that priority effects are stronger when organisms are under time constraints, so far priority effects under time constraints have not been studied (but see Johansson and Rowe (1999) and Sniegula et al. (2019) who studied time constraints and priority effects on cannibalism independently). Second, low ambient temperatures as experienced in high-latitude environments may also shape the strength of SMPE. Suboptimal low temperatures reduce foraging behaviour and hence decrease growth and development rates. Low temperatures can therefore reduce SMPE in thermally-constrained high-latitude populations (Rudolf and Singh 2013, Grainger et al. 2018).

For a good understanding on how SMPE shape an individual's fitness we need to understand the fitness-related physiological responses to the biotic and abiotic factors causing the priority effects (Poertner and Farrell 2008, Stoks et al. 2014). Environmental stress caused by biotic interactions (such as competition) and abiotic factors (such as time and thermal constraints) can strongly affect an organism's physiology (Flatt and Heyland 2011, Bozinovic and Pörtner 2015, Sniegula et al. 2017b). These effects on physiology may affect fitness and can occur as direct responses to stressors, or as indirect responses to compensatory life history responses to deal with stressors. For example, in insects poor-quality diet or food deprivation may directly cause a decrease in immune system activity (Campero et al. 2008, Triggs and Knell 2012), and increased temperatures may directly lead to a larger fat content and a decreased activity of key immunological parameters (Karl et al. 2011). As an example of an indirect effect, an increased development rate of time-constrained larvae may indirectly lead to increased oxidative damage under time constraints (Janssens and Stoks 2018). Nonetheless, life history and physiology may also be decoupled (Rolff et al. 2004, Karl et al. 2011, Laughton et al. 2017, Meister et al. 2017).

Here, we study how intraspecific competition mediated by hatching dates and seasonal time constraints affects life history and physiology through SMPE in the predatory and cannibalistic larvae of the damselfly *Lestes sponsa*. A previous study by Sniegula et al. (2019) showed that within a single population SMPE shape life history traits such as survival, growth and development rates in *L. sponsa*. The current study extends the one of Sniegula et al. (2019) by imposing time constraints and different temperatures on populations from different latitudes that have experienced different time constraints and temperatures for thousands of generations. This allows a first integrated test of both latitude-specific and photoperiod-mediated time constraints on SMPE, thereby also providing a rare test on geographic variation in SMPE. We hypothesise that 1) *L. sponsa* shows SMPE for life history and physiological traits. In particular, we expect advanced hatchers in mixed phenology groups, i.e. advanced hatchers reared together with non-advanced hatchers, to gain advantage over non-advanced ones with respect to food acquisition and hence increase predation rate (including cannibalism), and as a result will be able to increase development and growth rates, and their investments in immune function and energy storage (Johansson and Rowe 1999, Stoks et al. 2006, Yang and Rudolf 2010, Sniegula et al. 2017b). 2) Priority effects for life history and physiological traits will be more pronounced under the high-latitude thermo-photoperiod, i.e. under higher time constraints. This is because time constrained individuals are more competitive and cannibalistic towards conspecifics (Johansson and Rowe 1999, Sniegula et al. 2017a), which in turn should increase the magnitude of SMPEs. 3) Priority effects for life history and physiology will be more pronounced in northern populations, regardless of the rearing conditions. This is because the strongly time constrained northern individuals are more competitive and cannibalistic towards conspecifics (Sniegula et al. 2017a), and hence gain more advantage over non-advanced ones in food acquisition (Rasmussen et al. 2014).

Material and methods

We ran an experiment where high-latitude (strongly time constrained) and central-latitude (weakly time constrained) larvae were reared in groups in native and non-native combinations of temperatures and photoperiods (hereafter, thermo-photoperiods) (Fig. 1). By manipulating hatching date, we aimed to test whether early (advanced) hatchers take advantage over late (non-advanced) hatchers in terms of life history (survival, development and growth rates and mass at emergence). To get a more complete picture of the fitness implications of SMPE, we also tested for SMPE in three key fitness-related physiological parameters in insects: the activity of the enzyme phenoloxidase as a measure of the insect's immune function (González-Santoyo and Córdoba-Aguilar 2012), the body fat content as measure of long-term energy storage and protein content as a basic building component of insect tissues and muscles (Sterner and Elser 2002, Speight et al. 2008).

Study species

Lestes sponsa is a common Eurasian damselfly with a strictly univoltine life cycle (one generation/year). Adult females lay eggs during summer. About two weeks after egg laying, eggs enter winter diapause. Hatching takes place during the following spring, when water temperatures exceed 10°C. Along a latitudinal gradient, larval development and growth rates are adaptively adjusted by day length (photoperiod). A long photoperiod usually signals a late season and triggers larvae to accelerate growth and development, and this photoperiodic cue is stronger at high latitudes where the daylight period is longer (Johansson et al. 2010, Sniegula and Johansson 2010, Sniegula et al. 2014). The larvae of this species show cannibalistic behaviour (Sniegula et al. 2017a). The seasonal adjustments of life history traits, phenotypic and genetic variation in hatching dates (Sniegula et al. 2016a) and cannibalistic behaviour (Sniegula et al. 2017a), make *L. sponsa* a good species for studying SMPE across latitudes with more and less seasonally time constrained populations.

Collection and housing

To obtain eggs, adult mating females were collected in two central and three northern populations. The central populations were sampled on 29–30 July 2018 in north-western Poland (37 females from Czarne Wlk, 53°39′N, 16°16′E,
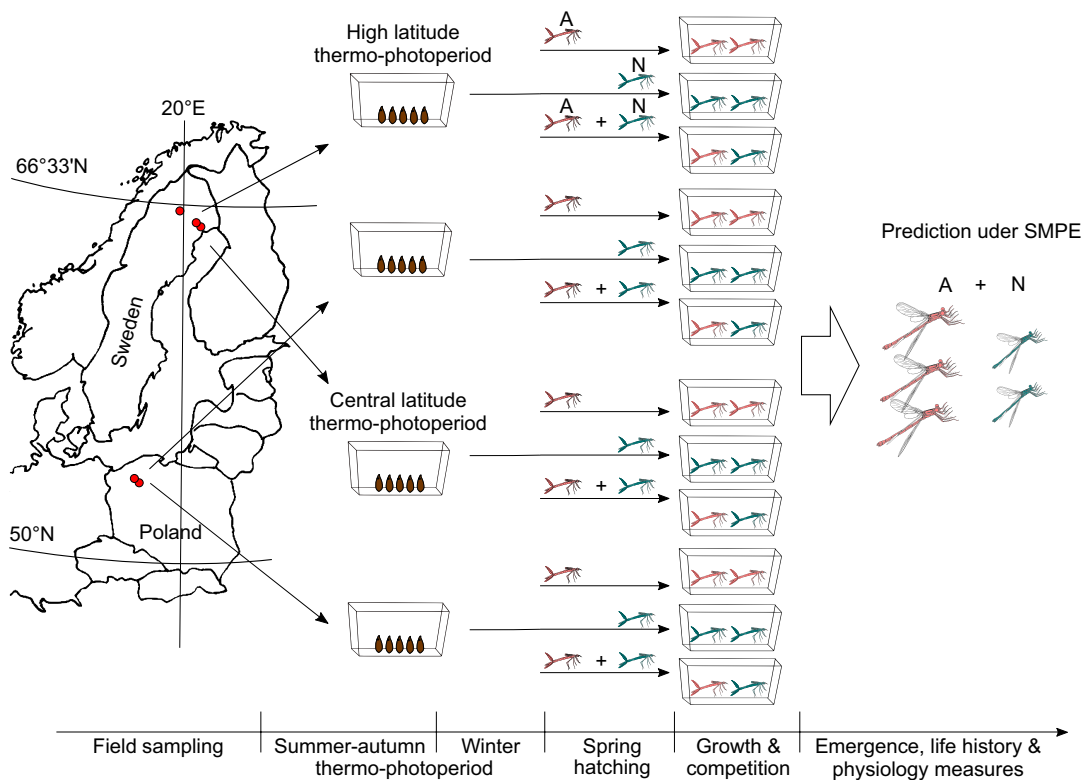

Figure 1. The full factorial experimental design to study size-mediated priority effects (SMPE). Damselfly eggs and larvae from both latitudes were reared in native and non-native thermo-photoperiods. Besides non-mixed advanced (A) and non-mixed non-advance (N) phenology groups, we also installed two other mixed phenology groups: advanced larvae in mixed phenology group (A+N) and non-advanced larvae in mixed phenology group (N+A), creating four phenology groups. At emergence, we quantified life history and physiology traits. The time scale is arbitrary and does not represent the length of each experimental stage. SMPE would be present when A+N individuals showed higher values of life history and physiological measures over N+A group and non-mixed A and N group.

and 43 females from Nadarzyce, 53°29′N, 16°30′E). The northern populations were sampled on 1 August 2018 in northern Sweden (31 females from Boden, 65°51′N, 21°27′E, 32 females from Luleå, 65°36′N, 22°7′E and 13 females from the Luleå Airport, 65°31′N, 22°8′E). Females were placed in plastic jars with wet filter paper for egg laying. On 2–3 August 2018 central and northern egg clutches were transported to the Institute of Nature Conservation PAS in Krakow, Poland, by car (central region) and by plane (northern region). Upon arrival to the laboratory, every egg clutch was divided into two halves and put in separate plastic containers (15 × 10 cm, height 6.5 cm) filled with 400 ml of dechlorinated tap water. Half of the containers were placed in an incubator with the temperature and photoperiod (thermo-photoperiod) corresponding to summer at the central latitude. The other half of the containers were placed in an incubator with the thermo-photoperiod corresponding to the northern latitude (Supporting information). This resulted in a fully crossed experimental design (for sample sizes and for the experimental set up in climate incubator see the Supporting information). The eggs remained in these thermo-photoperiod conditions until they were 18 days old. Thereafter, they were transferred to an incubator with a lower temperature and shorter day length to successively simulate the conditions of late summer, fall and winter (Supporting information). Hatching dates were manipulated by varying the length of the winter conditions, i.e. by changing the dates of initiation of spring conditions.

During the initiation of spring conditions, egg clutches from the same population were pooled. We created two hatching groups: advanced (A) and non-advanced (N). The advanced group of eggs was retrieved from winter conditions on 20 November 2018; non-advanced eggs were retreated from winter conditions on 4 December 2018. These dates were chosen to ensure there was a difference in size between larvae from two hatching groups. Such time difference in hatching dates is relevant to what happens in nature, and this because some individuals react stronger than others to increasing water temperature (Sniegula et al. 2016c) and post-diapause egg development and hatching date is temperature, not photoperiod, dependent (Norling 2018). In addition, temperature might vary in a pond since some areas are shady and some are sunny. This difference in shade cause differences in temperature at a quite small scale of meters. The difference in temperature between these areas cause differences in hatching dates. Larvae from these areas might later on disperse and cause a mixture of larvae with different ages and sizes, creating an opportunity for size-mediated competition. What is more, the difference in hatching dates between A and N groups lays within a range of hatching dates that was early shown by *L. sponsa* in laboratory conditions (Sniegula et al. 2016c). At hatching, larvae were randomly chosen from the two hatching groups to establish four experimental phenology groups: advanced (A), non-advanced (N), mixed-phenology advanced (A+N) and mixed-phenology non-advanced (N+A). A+N and N+A groups were reared in shared containers, but were considered as separate groups (= levels) in statistical analyses. We assumed the sex ratio at the beginning of the experiment was 1:1 (Corbet 1999). The mixed phenology groups consisted of individuals that differed in hatching dates and hence size. The difference between hatching dates was on average 15 days (range: 14–16 days due to temporal variation in hatching dates). Advanced larvae hatched on 19–20 December 2018, and non-advanced on 3–5 January 2019. The temperature and photoperiod at these hatching dates matched 3 May and 24 May 2019 for central advanced and non-advanced individuals, respectively, and 14 June and 5 July 2019 for northern advanced and non-advanced individuals, respectively. We used these hatching dates because *L. sponsa* starts hatching early May in central-latitude sites and early June in high-latitude sampling sites (Sniegula et al. 2019; Sniegula, unpubl.). At the central-latitude site the average daily temperatures during these dates reach 11°C (unpubl.). However, earlier hatching (by a couple weeks) is possible at latitudes similar to our sampling latitudes (Jödicke 1996). The thermo-photoperiods were changed weekly (every Friday) in accordance to Flake model estimates (described below; Supporting information). The photoperiod included morning and evening Civil twilight. In both thermo-photoperiods hatching occurred within two days. Larvae from each population per region were picked at random and put in containers (16 × 12 cm, height 8 cm) filled with 600 ml of dechlorinated tap water. In A and N phenology groups, a total of 16 larvae were placed in each container. In mixed phenology groups (A+N and N+A) we first placed eight advanced larvae, and after 15 days we added eight non-advanced larvae. The larval density used in this experiment is higher than the densities observed in natural populations of *Lestes* sp.: 106 larvae per m² in the field (Duffy 2011) versus 833 larvae per m² in the experiment, but within a range of larval densities in other damselfly species recorded in nature (Corbet 1999, p. 611). We used high larval density to increase the encounter rate and hence the impact of size-mediated priority effect on measured traits (cf. Sniegula et al. 2019). In the mixed phenology groups, when non-advanced larvae were added to advanced larvae, the latter had gone through two moults (Sniegula et al. 2019). The number of containers per phenology group and thermo-photoperiod were 12–14 (details are given in the Supporting information). Throughout the experiment, larvae were fed with laboratory-cultured *Artemia* nauplii. Phenology groups that started with 16 larvae (A and N) received 10 rations of 215 (SE=7, n=10) nauplii per day. Before every feeding we noticed a left-over of *Artemia* nauplii, suggesting that the primary prey for damselfly larvae were provided ad libitum. Before the introduction of non-advanced individuals in mixed phenology groups, advanced individuals received half of the food rations, i.e. five rations. The ratio was doubled when non-advanced individuals were introduced in mixed phenology groups (A+N and N+A). By changing the food ratio in mixed phenology groups, we could compare all the phenology groups equally in terms of food provided per individual larva. As a surrogate for natural vegetation three nylon nets forming a cross were placed in each container.

Temperatures were derived from the Lake Model FLake (Lake Model FLake 2009). The model realistically describes shallow water temperatures thorough the season (Dinh Van et al. 2014) – a common habitat for *L. sponsa* larvae (Corbet 1999). The model considers surface thermal radiation, solar radiation, wind speed, dew point temperature and air temperature to calculate weekly estimates of water temperatures at a given depth (Lake Model FLake 2009). However, the model does not consider temperature inversion that impacts surface temperatures considerably at higher latitudes (Corbet 1969). Therefore, based on field measurements (SS, unpubl.), and to better represent water temperature that the damselfly larvae experience in the wild, we increased the temperature predicted by this model by 2°C.

To distinguish between advanced and non-advanced larvae in the A+N group, larvae were marked by cutting a tibia of the right or left middle leg. This marking persists until emergence and does not influence the life-history traits measured during this experiment (Hagler and Jackson 2001, Sniegula et al. 2019). Legs were cut when larvae were 28–29 days old. The same treatment was given to larvae in A and N groups to exclude potential differences between groups caused by marking.

Newly emerged adults were placed in 200 ml plastic cups covered with mosquito net. The day after emergence, adults were frozen at −80°C. Frozen adults were transported on dry ice to the Laboratory of Evolutionary stress ecology and Ecotoxicology (Univ. of Leuven, Belgium) for physiological analyses.

Response variables

Life history
Four life history traits were estimated: larval age at emergence, mass at emergence, larval growth rate and survival until emergence. Age at emergence was quantified as the number of days between egg hatching and adult emergence. The day after emergence, adult wet mass was measured to the nearest 0.01 mg using an electronic balance. The larval growth rate was estimated by dividing adult mass at emergence by larval development time. Survival until emergence was determined by counting the number of individuals that emerged. Mortality caused by cannibalism was distinguished from mortality caused by other reasons than cannibalism (e.g. developmental errors) by monitoring missing larvae or dead larvae with missing body parts (other than the tibiae of the left or right middle legs that were cut for marking).

Physiology
For the physiological analyses, damselfly bodies (excluding wings and legs) were ground together with phosphate buffer solution (PBS, 15 μl for each milligram of wet mass) and centrifuged (at 10 000 g for 5 min at 4°C). Physiological analyses of total body fat and total protein contents, and phenoloxidase activity were done on the homogenates.

The total body fat was assayed based on the protocol by Marsh and Weinstein (1966), that was optimised for the

damselfly adults. For this, 8 μl of the homogenate was combined with 56 μl 100% sulphuric acid in glass tubes, and heated for 20 min at 150°C. When tubes cooled down, 64 μl Milli-Q-Water was added (1:1 ratio). 30 μl of the prepared sample was put in a well of a 384-well microliter plate to measure absorbance at 340 nm (three technical replicates for each sample, with the averaged value used in statistical analyses). The measurements were made on an Infinite M2000 (TECAN) plate reader. To convert absorbances into fat content, the standard curve of glyceryl tripalmitate was used.

The total body protein content was determined following the Bradford (1976) method. Briefly, 1 μl of original homogenate with 160 μl of Milli-Q-Water. After adding 40 μl of Bio-Rad Protein Dye the sample was mixed. The absorbance was measured at 595 nm after 5 min of incubation at 25°C. Absorbances were converted into protein contents using standard curves of bovine serum albumin. For statistical purposes the average of three technical replicates was used.

The phenoloxidase activity was measured using a modified version of the procedure by Stoks et al. (2006). For this, 10 μl of homogenate was combined with 10 μl of PBS buffer (phosphoric buffered saline), and mixed with 5 μl of chymotrypsine in wells of a 384-well microtiter plate. Afterwards, the mixture was incubated for 5 min at room temperature. After incubation, the substrate L-DOPA (1.966 mg Dihydroxyphenyl-L-alanine per 1 ml of PBS-buffer) was added and mixed with the samples. Thereafter, the linear increase in absorbance at 490 nm was measured every 20 s for 30 min (at 30°C). The activity was quantified as the slope of the reaction curve. The average was taken from the two technical replicates of each sample.

Statistical methods

For statistical analyses we used R ver. 3.6.1 (<www.r-project.org>). In all models, we used Wald-χ^2 statistics (ANOVA type II in car package) to test for fixed effects (Fox and Weisberg 2011). For survival until emergence a generalized linear mixed model with binomial error distribution (glmer function from lme4 package) was used. The other life history (development time, body mass and growth rate) traits and the physiological traits (fat content, protein content and phenoloxidase activity) were analysed using linear mixed models [lmer function from lme4 package (Fox and Weisberg 2011)]. Region (central and northern), phenology group (A, N, A+N and N+A), thermo-photoperiod (high- and central-latitude combination of temperature and photoperiod) and sex were added as explanatory variables. Population within region and container within population were considered random effects. Initially, we ran full models that included all main effects and interaction terms. Thereafter, the interaction terms with p-value $\geq$ 0.1 were removed (Leek and Peng 2015). In case of significant interaction terms, we assessed the significance of the differences between treatment levels using the emmeans function (Lenth et al. 2020). When analysing PO activity, we used protein content as a covariate, and for fat content we used body mass as a covariate. Priority effects on life history

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or physiology would be indicated by a significant phenology group term whereby the trait value in the A+N group would be higher (mass at emergence, growth rate, fat content, protein content and phenoloxidase activity) or lower (development time) than in the other phenology groups. These higher or lower trait values in A+N group compared to non-mixed A and N groups would indicate an advantage for A+N larvae caused by their earlier hatching in the presence of non-advanced, later hatched N+A larvae. This likely operates through the size advantage of A+N larvae compared to N+A larvae that is expected to create an advantage in intraspecific competition, potentially leading to SMPEs (Fig. 1).

Results

In total 203 (8.6%) larvae likely died due to intrinsic mortality, i.e. by other reasons than cannibalism. Intrinsic mortality did not differ between phenology groups, regions and thermo-photoperiods ($\chi^2 \leq 0.186$, $p \geq 0.67$) and hence did not affect other analyses on life history and physiological traits. The other 1321 (55.8%) larvae died due to cannibalism. The remaining 844 (35.6%) survived until emergence. Except for survival until emergence, non-advanced larvae in mixed phenology group (N+A) was excluded from the analyses because of low number or absence of individuals surviving until emergence.

Life history

There was a priority effect for larval survival until emergence. Advanced larvae in mixed groups (A+N) had the highest survival, while s (N+A) had the lowest survival in both thermo-photoperiods. On average, larvae had ca 19% higher survival in the central than in the high-latitude thermo-photoperiod, and survival was ca 14% higher in the central than in the northern region of origin (based on a comparison of numbers of survived larvae/total in the group; Supporting information), yet there was no phenology × thermo-photoperiod interaction (Fig. 2a, Table 1). In both regions, females had higher survival than males, especially in the central region (region × sex interaction; Supporting information, Table 1). Note that few individuals from N+A group survived until emergence. To have more balanced statistics with respect to sample size in different phenology groups, we ran additional test using number of individuals that survival until day 42 (advanced larvae) and day 28 (non-advanced larvae) – the time of larval marking by leg-cutting. In this analysis we excluded sex because at this larval stage we could not distinguish sex. The results were qualitatively the same as for larval survival until emergence (results not shown), indicating significant priority effects in survival at two time points of larval development.

There was no priority effect for age at emergence, i.e. development time. Advanced non-mixed phenology larvae (A) emerged later than non-advanced non-mixed phenology larvae (N), but only in central latitude thermo-photoperiod (phenology × thermo-photoperiod interaction; Fig. 2b, Table 1, Supporting

information). Northern larvae emerged earlier than central larvae (Fig. 2b, Table 1, Supporting information). Males emerged earlier than females (Table 1, Supporting information).

There was a trend for a priority effect for mass at emergence, but only in the central latitude thermo-photoperiod and in central damselflies. Indeed, advanced larvae in mixed groups (A+N) had a higher mass than advanced larvae from non-mixed groups (A), but only in the central latitude thermo-photoperiod and in central damselflies (phenology × region × thermo-photoperiod interaction, Fig. 2c, Table 1, Supporting information). This suggests that non-advanced/late season hatching and the high latitude thermo-photoperiod (= time constraint) removed a priority effect in central damselflies. Damselflies reared in the high-latitude thermo-photoperiod had a higher mass, indicating that time constraints increased the investment into mass (Fig. 2c, Table 1). Regions did not differ in mass. Females were heavier than males (Table 1, Supporting information).

There was no priority effect for growth rate. Advanced central larvae in mixed groups (A+N) grew faster than advanced central larvae in non-mixed groups (A), while no such difference was found in northern larvae (phenology × region interaction; Supporting information, Table 1). Larvae grew faster in the high-latitude thermo-photoperiod. Northern larvae grew faster than central larvae (Supporting information, Table 1). Females grew faster than males with northern females having the highest growth rate; central males and females grew at the same rate (region × sex interaction; Supporting information).

Physiology

There was no priority effect for PO activity. Northern damselflies had a lower PO activity in the high-than in central-latitude thermo-photoperiod; central damselflies did not show such difference (thermo-photoperiod effect and region × thermo-photoperiod, Fig. 3a, Table 1, Supporting information). Advanced females from the non-mixed phenology group had a higher PO activity than non-advanced males from the non-mixed phenology group, and advanced males from the mixed phenology group had a higher PO activity than non-advanced males from the non-mixed phenology group (phenology × sex interaction; Supporting information). Overall, there was a negative correlation between PO activity and larval growth rate ($r = -0.19$, $p < 0.001$).

No priority effect was found for fat content. Northern damselflies had a higher fat content in the high-than the central latitude thermo-photoperiod, while no difference was observed in central damselflies (region × thermo-photoperiod interaction; Fig. 3b, Table 1, Supporting information). Males had a higher fat content than females (Table 1, Supporting information).

No priority effect was found for adult protein content. The high latitude thermo-photoperiod led to a higher protein content. Central larvae had higher protein content than northern larvae, but only under the central latitude thermo-photoperiod (interaction region × thermo-photoperiod; Fig. 3c, Table 1). Under central latitude thermo-photoperiod, central males had higher protein content than
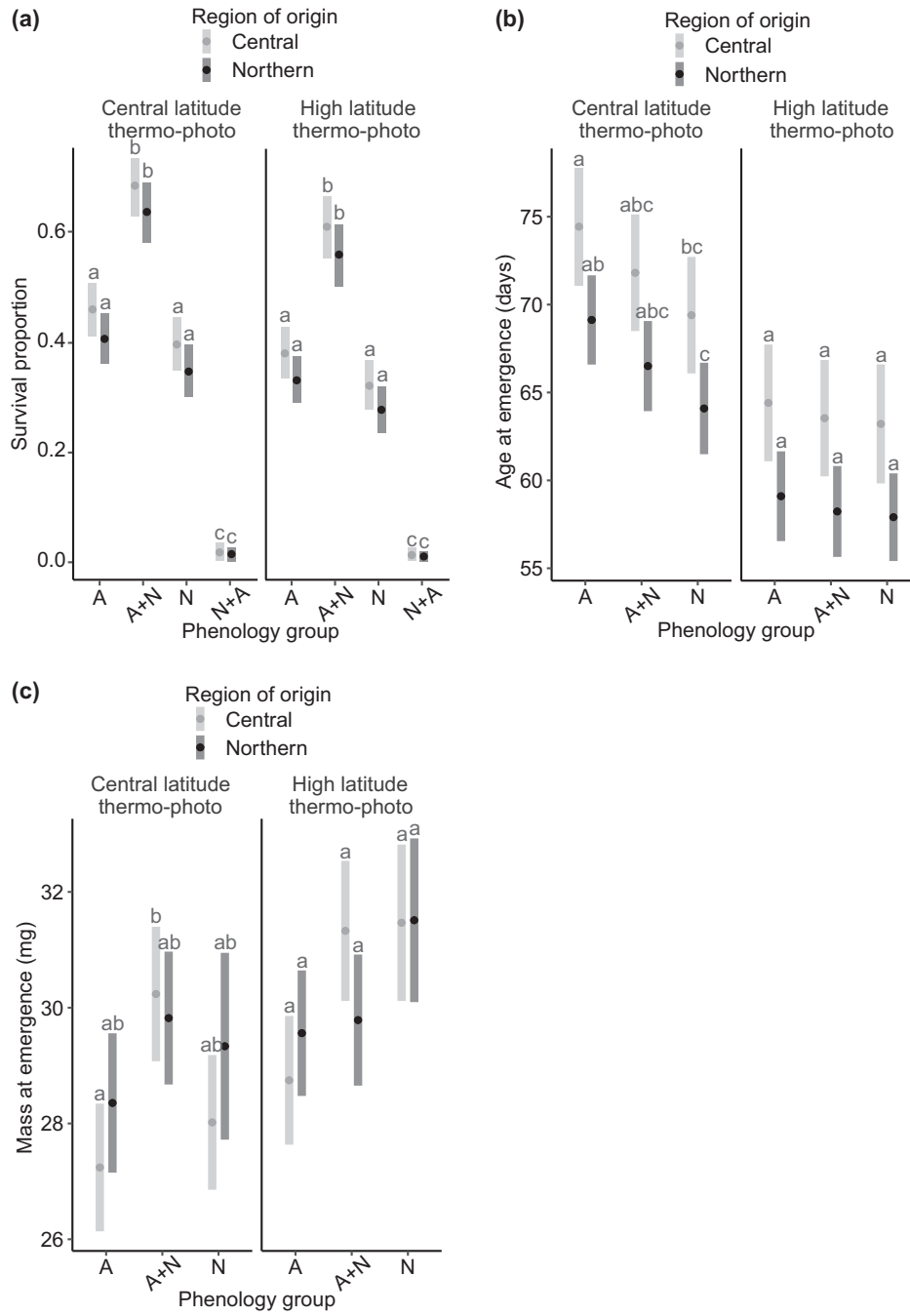
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Figure 2. (a) Proportions of survival until emergence, (b) age at emergence and (c) mass at emergence across different regions (central and northern), phenology groups (A, A+N, N and N+A) and thermo-photoperiods (central- and high latitude) in *L. sponsa*. Different letter codes indicate significant differences between the phenology groups and regions within a given thermo-photoperiod based on contrast comparisons. Error bars indicate estimated 95% CI. The individuals of the phenology groups are coded as follows: A are advanced individuals in a non-mixed phenology group, A+N are advanced individuals in a mixed (advanced and non-advanced) phenology group, N are non-advanced individuals in a non-mixed phenology group, N+A are non-advanced individuals in a mixed phenology group. Except for the proportion of survival (a), N+A group was excluded from the analyses and figures because of the low sample size.

Table 1. Results from mixed models on life history and physiological traits. The *L. sponsa* phenology group (A, A+N, N and N+A for survival and emergence success, and A, A+N and N for the rest of response variables analysed), region (populations from central and northern latitudes), thermo-photoperiod conditions (combined temperatures and photoperiods matching high and central latitudes) and sex were fixed effects. Population and container were random effects. Interaction terms with p-values ‹ 0.1 were removed from the final models and are not shown in the table. Except for the survival until emergence, N+A group was excluded from the analyses because of the low sample size. P-values close or lower than 0.05 are bolded for clarity.

Predictor	df	χ^2	p
Survival until emergence			
Phenology	3	176.65	**< 0.001**
Region	1	5.83	**0.016**
Thermo-photoperiod	1	11.93	**< 0.001**
Sex	1	46.93	**< 0.001**
Region × sex	1	11.12	**0.001**
Age at emergence			
Phenology	3	18.97	**< 0.001**
Region	1	13.76	**< 0.001**
Thermo-photoperiod	1	190.73	**< 0.001**
Sex	1	4.68	**0.03**
Phenology × thermo-photoperiod	2	6.49	**0.039**
Mass at emergence			
Phenology	2	23.42	**< 0.001**
Region	1	0.45	0.504
Thermo-photoperiod	1	18.54	**< 0.001**
Sex	1	89.65	**< 0.001**
Phenology × region × thermo-photoperiod	2	16.56	**0.02**
Growth rate			
Phenology	2	23.3	**< 0.001**
Region	1	22.25	**< 0.001**
Thermo-photoperiod	1	105.19	**< 0.001**
Sex	1	27.91	**< 0.001**
Phenology × region	2	4.62	0.099
Region × sex	1	5.03	**0.025**
Phenoloxidase			
Phenology	2	3.45	0.178
Region	1	0.0002	0.989
Thermo-photoperiod	1	15.33	**< 0.001**
Sex	1	2.15	0.142
Region × thermo-photoperiod	1	3.73	**0.053**
Phenology × sex	2	7.44	**0.0242**
Fat			
Phenology	2	0.93	0.82
Region	1	0.4	0.53
Thermo-photoperiod	1	2.30	0.13
Sex	1	38.93	**< 0.001**
Region × thermo-photoperiod	1	10.00	**0.002**
Protein			
Phenology	2	3.59	0.17
Region	1	6.09	**0.01**
Thermo-photoperiod	1	71.47	**< 0.001**
Sex	1	2.28	0.13
Region × thermo-photoperiod	1	9.5	**0.002**
Phenology × sex	2	9.41	**0.009**
Phenology × region × sex	5	13.75	**0.02**
Region × thermo-photoperiod × sex	2	7.35	**0.03**

northern males (region × thermo-photoperiod × sex interaction, Supporting information). Central males had a higher protein content than northern males, but only in the mixed phenology group (A+N) (phenology × region × sex interaction, Supporting information).

Discussion

Our results support our first hypothesis that advanced hatchers assembled with non-advanced hatchers show size-mediated priority effects (SMPEs) for survival until emergence. Indeed,
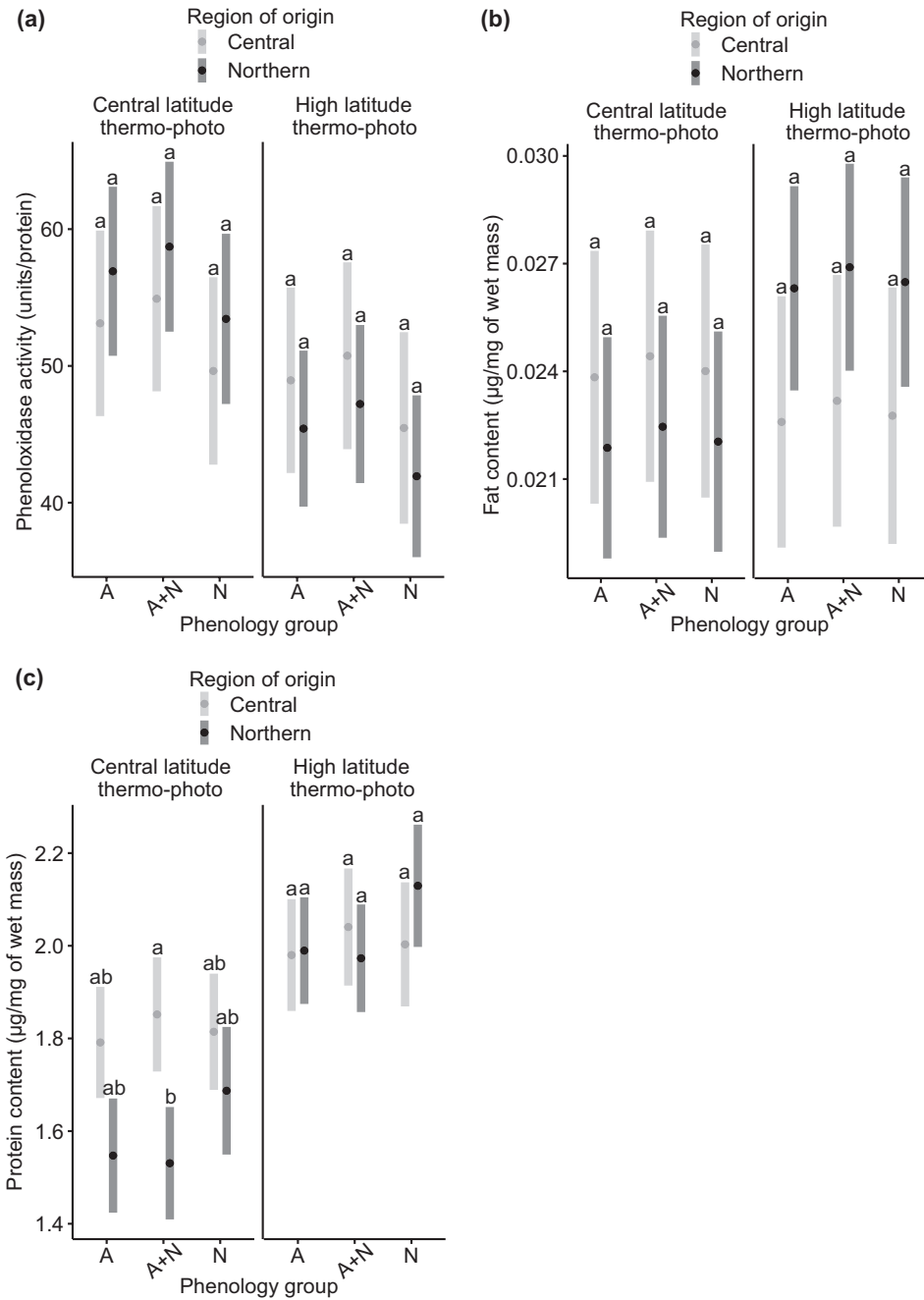
Figure 3. (a) Phenoloxidase activity (PO), (b) fat content and (c) protein content across different regions (central and northern), phenology groups (A, A+N and N) and thermo-photoperiods (central- and high latitude) in *L. sponsa*. Different letter codes indicate significant differences between the phenology groups and regions within a given thermo-photoperiod based on contrast comparisons. Error bars indicate estimated 95% CI. Abbreviations as in Fig. 2.

advanced-hatched *Lestes sponsa* larvae reared in mixed phenology groups showed the highest survival, while non-advanced hatched larvae reared in mixed phenology group – the lowest survival. Yet, this hypothesis was not supported for growth and development rates, and physiology. With few exceptions, we found no support for our second and third hypotheses that

SMPEs should be stronger under the high-latitude thermophotoperiod or in high-latitude populations. In these cases, priority effects were instead apparently outweighed by seasonal time constraints. The results suggest that SMPEs and seasonal time constraints shape life history traits in a non-additive way, and have little or no effects on physiological traits.

Survival until emergence represent a major component of fitness that is shaped by ecological challenges posed by the environment (Stearns 1992). We found that the larger advanced-hatched larvae reared in assemblage with smaller non-advanced hatchers had the highest survival due to their increased cannibalism on non-advanced hatchers, leaving very few non-advanced larvae alive at emergence. This supports previous findings that intraspecific predation is determined by asymmetry in larval size, and that such size-specific interaction can shape population structure (Anholt 1994, Suhling and Lepkojus 2001, Eitam et al. 2005, Jara 2014, Sniegula et al. 2019) and thus be considered as a priority effect (Yang and Rudolf 2010). However, the results did not support our second hypothesis that SMPE should be stronger under time constraints because time constrained individuals are more competitive towards conspecifics as well as more effective in prey capture (Johansson and Rowe 1999, Sniegula et al. 2017a, Golab et al. 2020). Indeed, there was no statistical interaction between hatching phenology and the thermo-photoperiod treatment for survival (Table 1). A possible explanation for the absence of thermo-photoperiod effects on SMPE for survival rate is that *L. sponsa* larvae show little phenotypic plasticity in antagonistic interactions in response to environmental constraints such as temperature. Indeed, Sniegula et al. (2019) showed that advanced and non-advanced hatched *L. sponsa* assemblages responded in the same way in their antagonistic interactions at the different temperatures. Nevertheless, our results show also that cannibalism increased in all phenology groups when larvae were time constrained, i.e. had a northern thermo-photoperiod, confirming previous results in the study species (Sniegula et al. 2017a). This shows that larvae compensate for time constraints by increasing predation rate and that this compensation is not confined by suboptimal high-latitude temperatures. Interestingly, SMPE in survival until emergence did not translate into SMPE in other life history and physiological traits.

Age and mass at emergence are important fitness traits in temperate ectotherms with complex life cycles (Nylin and Gotthard 1998, De Block and Stoks 2005, Sniegula et al. 2018), and SMPE may strongly shape these traits (Eitam et al. 2005). Advanced *L. sponsa* larvae in the mixed phenology group did not, however, emerge earlier or gain a higher mass at emergence than other phenology groups (there was however a trend for SMPE for mass at emergence in central damselflies reared in native central latitude thermo-photoperiod, Fig. 2c). This is surprising as these larvae showed the highest cannibalism rate, hence could have benefited from a higher food availability (Jefferson et al. 2014). This result is in contrast from a previous experiment where central latitude *L. sponsa* larvae were reared in their native photoperiod (indication of intermediate time constraints) at a constant temperature, and did show SMPEs for development time and mass at emergence (Sniegula et al. 2019). Thus, for these life history traits seasonal constraints in terms of combined time and thermal restraints seem to remove priority effects. Similar larval responses in life history traits to time, but not thermal, constraints were shown

in previous studies where organisms were reared individually, i.e. in the absence of intraguild competitors (Johansson et al. 2001, Sniegula et al. 2014, Mikolajewski et al. 2015). This suggests that SMPE plays a minor role in shaping age and mass at emergence under time-constrained conditions. To sum up, we found no support for our second and third hypotheses that SMPE are more pronounced under the high-latitude compared to the low-latitude thermo-photoperiod or in the northern compared to the southern populations. Nevertheless, larvae raised at time constrained conditions did show a faster development as has been shown in previous studies (reviewed in Dmitriew 2011, Sniegula et al. 2014).

Interestingly, larvae reared in time constrained high-latitude conditions invested more into mass at emergence than larvae from less time constrained conditions. This shows that time constraints did not intensify the trade-off between age and mass at emergence, as it would be expected from the theory (Rowe and Ludwig 1991) and previous empirical studies (Johansson et al. 2001, Shama and Robinson 2006, Mikolajewski et al. 2013, Sniegula et al. 2016b). The lack of a tradeoff between age and size at emergence might be explained by a flexible growth rate (Abrams et al. 1996). Indeed, the growth rate in the high latitude thermo-photoperiod overcompensated the shorter development times under time stress. We suggest that the overcompensation happened due to improved conditions with respect to prey quantity, i.e. a thinning effect, and quality, i.e. the presence of conspecific prey as an addition to zooplanktonic *Artemia* nauplii (Johansson et al. 2001, Brodin and Johansson 2002, Sniegula et al. 2017a). A mechanistic explanation could be an increased activity and effectiveness in prey capture under time constraints, as shown in another experiment on *L. sponsa* (Golab et al. 2020). The here observed absence of SMPE in age and mass at emergence might be found in nature under conditions where prey availability is high. In damselflies, the highest cannibalism rate, usually occur early during the development rate (Anholt 1994, Sniegula et al. 2017a) when larval densities are the highest (Corbet 1999, Duffy 2011). The absence of SMPEs in environments with high prey abundance has been theoretically predicted (Abrams 1991). However, the intensity of SMPEs in nature under conditions where prey abundance is limited remains unknown. Finally, the strictly univoltine life cycle of *L. sponsa* could also play a role in the overcompensation explanation, since this species cannot take a second year in the larval stage and thus has to emerge before the summer season ends (cf. Sniegula et al. 2012).

A high cannibalism rate can lead to changes in population structure (Jara 2014, Toscano et al. 2017). We suggest that cannibalism may cause a female-biased population structure because fewer males than females survived until emergence, assuming an equal sex ratio at hatching (Corbet 1999, p. 250). Female larvae need to invest more resources into growth because they reach a larger size and mass at emergence than males (Johansson et al. 2005, Hyeun-Ji and Johansson 2016, Sniegula et al. 2016b; current study), and larger female size is linked to increased fitness (Stearns 1992, Sokolovska et al. 2000). In addition, *L. sponsa* males experience stabilizing

selection for intermediate size (Stoks 2000), likely due to an advantage of smaller males in scramble competition. Hence, male size is not under directional selection. We therefore suggest that females imposed stronger non-lethal interference competition and cannibalism on males due to sexual size dimorphism shaped by sexual selection, however, with no indication of sex-biased SMPEs.

We did not find that SMPE affected energy storage in terms of fat content and major building blocks of other than fat tissues, including muscles, in terms of protein content at emergence. Despite this, high-latitude larvae gained more energy storage and protein content than central-latitude larvae when grown in the time constrained high-latitude thermo-photoperiod. We suggest that these higher levels could be due to a higher cannibalism rate among northern larvae, especially under time constrained conditions, as shown in the current experiment and previous studies on the damselfly (Sniegula et al. 2017a) and other insects (Lund et al. 2016, Gillespie et al. 2020). These results also indicate no expected costs of fast development and growth in terms of a reduced fat and protein contents at emergence (Wieser 1994). Such cost was found in previous studies on other damselflies where larvae were grown individually (Stoks et al. 2006a, Verheyen et al. 2018). In our case, an increased food intake likely offset this physiological tradeoff (cf. van Noordwijk and de Jong 1986), but increased ecological risk in terms of conspecific predation risk (cf. Sniegula et al. 2017b; this paper). A higher energy storage at emergence is known to increase insect survival and dispersal capacity during the teneral stage (Braune and Rolff 2001, Dmitriew and Rowe 2007, Scott et al. 2007). Although we are not aware of long-term studies focusing on cross-metamorphic effects of protein content in damselflies (Stoks and Córdoba-Aguilar 2012), a higher investment into proteins during the larval stage may have a range of positive effects during adult stage, including an increased physiological body condition (Nation 2008) and increased adult flight performance (Therry et al. 2014). Note that in adult odonates up to 60% of their body mass is flight muscle (Marden 1989).

Also for PO activity at emergence we did not find a SMPE. Phenoloxidase activity is a key physiological trait involved in the host response to immune challenges (González-Santoyo and Córdoba-Aguilar 2012). We expected that the adult immune function expressed as PO activity to be higher in early-hatched individuals. Instead, time constraints imposed by the high-latitude thermo-photoperiod reduced PO activity, and this especially in northern damselflies. We suggest that decreased investment in immune function was caused by a tradeoff between on the one hand larval development and growth rate, and on the other hand PO activity, and this especially in northern larvae as northern individuals showed in general a faster development and growth than central larvae. A similar immune cost of fast development induced by seasonal time constraints was found in the congeneric damselfly *L. viridis* (Rolff et al. 2004, Stoks et al. 2006a, Janssens and Stoks 2018), which, like *L. sponsa*, is strictly univoltine and has a fast life style (Corbet 1999, Johansson 2000). Such

physiological cost of increased development and growth may be buffered in species with slow life style characteristics (Mikolajewski et al. 2015). It is worth mentioning that accelerated larval development can cause negative changes in other physiological and life history parameters, e.g. oxidative damage to lipids, proteins and DNA and adult life span (Janssens and Stoks 2018), which may be relevant in *L. sponsa*. However, this needs further investigation.

To conclude, we documented strong SMPEs for survival that further suggest that SMPEs may strongly affect population structure (Yang and Rudolf 2010, Takashina and Fiksen 2020). We made two important additions to our current insights in SMPEs. First, while we detected SMPEs for survival, this was not the case for growth and development rates, and physiology (energy storage, protein content and investment in immune function). This trait-dependence of SMPEs could be explained by different trait responses to seasonal time constraints. In traits for which SMPE did not appear to happen, priority effects were apparently outweighed by seasonal time constraints. Second, we explored the context-dependency of SMPEs thereby showing these to be not more pronounced for survival under experimentally manipulated (thermo-photoperiod) or latitude-associated time constraints. Hence, our study thereby makes an important addition to geographic variation in SMPEs, a largely neglected topic (but see Young et al. 2015).

Author contributions

Mateusz Raczyński: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Software (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Robby Stoks**: Formal analysis (supporting); Methodology (supporting); Software (equal); Writing – original draft (supporting); Writing – review and editing (supporting). **Frank Johansson**: Writing – original draft (supporting); Writing – review and editing (supporting). **Szymon Sniegula**: Conceptualization (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal).

Data availability statement

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.hdr7sqvj6> (Raczyński et al. 2021).

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PAPER 3

Warming and predation risk only weakly shape size-mediated priority effects in a cannibalistic damselfly

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Warming and predation risk only weakly shape size-mediated priority effects in a cannibalistic damselfly

Mateusz Raczyński[1✉], Robby Stoks[2] & Szymon Sniegula[1✉]

Differences in hatching dates can shape intraspecific interactions through size-mediated priority effects (SMPE), a phenomenon where bigger, early hatched individuals gain advantage over smaller, late hatched ones. However, it remains unclear to what extent and how SMPE are affected by key environmental factors such as warming and predation risk imposed by top predators. We studied effects of warming (low and high temperature) and predation risk (presence and absence of predator cues of perch) on SMPE in life history and physiological traits in the cannibalistic damselfly *Ischnura elegans*. We induced SMPE in the laboratory by manipulating hatching dates, creating following groups: early and late hatchlings reared in separate containers, and mixed phenology groups where early and late hatchlings shared the same containers. We found strong SMPE for survival and emergence success, with the highest values in early larvae of mixed phenology groups and the lowest values in late larvae of mixed phenology groups. Neither temperature nor predator cues affected SMPE for these two traits. The other life history traits (development rate and mass at emergence) did not show SMPE, but were affected by temperature and predator cues. A tendency for SMPE was found for protein content, in the high temperature treatment. The other physiological traits (phenoloxidase activity and fat content) showed fixed expressions across treatments, indicating decoupling between physiology and life history. The results underline that SMPEs are trait-dependent, and only weakly or not affected by temperature and predation risk.

One of the biggest challenges in ecology is to understand and predict the impact of climate change on species and populations[1,2]. However, such understanding is complicated by the fact that species are embedded in complex communities. Therefore, it is not enough to understand how species are affected by warming per se, but also how warming changes their antagonistic[3,4] and synergistic[5,6] interactions. Changes in temperature have indeed been shown to affect antagonistic interactions between organisms[7–10]. These changes in interactions can be caused by shifts in phenological events[11], for example, by changes in relative hatching dates among interactive organisms[12,13]. In predator–prey systems, higher temperatures may lead to increased activity and encounter rates that benefits predators in terms of higher food acquisition, earlier time at maturity and larger final size[14,15] through changes in physiology[16,17].

Specifically for cannibalistic interactions, the outcome of intraspecific encounters is strongly determined by the difference in body size[18–20], and encounter rates and cannibalism rates increase under warming[21,22]. In such situations, larger individuals take advantage over smaller individuals leading to so called size-mediated priority effects, SMPE[14]. It has been shown that individuals that appear early in a habitat show SMPE in life history (e.g. increased adult mass and survival) and in physiological traits (e.g. increased metabolic rates and improved immune function)[23–25]. There is accumulating evidence that changes in phenological events such as relative hatching dates increase the magnitude of SMPE due to enlarged variation in relative body size of interacting animals[9,26]. Although the theoretical backgrounds of priority effects in a warming world have been explored[4,27], to our knowledge there have been few empirical approaches that linked life history with physiology in the context of SMPE caused by temperature-mediated hatching dates[28,29].

Predator–prey interactions can have direct consumptive, and indirect non-consumptive effects. Non-consumptive predator effects refer to reductions in prey fitness through behavioural and physiological changes[30–33].

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The direction and intensity of non-consumptive predator effects may vary depending on the characteristics of the predator–prey couple, for example the predator:prey body size ratio which determines predator gape limitation[34]. Non-consumptive predator effects caused by visual or chemical predator cues can lead to reduced prey activity, food intake and growth[35–38]. Instead, prey may also increase growth rate to escape gape-limited predators[39], but this often leads to costs in terms of a decreased size at maturity[40] and a reduced ability to neutralize free radicals[41,42]. Non-consumptive predator effects can have equally or even more negative consequences for prey communities than consumptive effects[43,44]. However, it is still unclear whether and how the non-consumptive predator effects impact SMPE in prey, especially when prey represent intermediate, cannibalistic predators in a food chain, and the predators are at the top of the food chain. Furthermore, the presence of predator cues may change the effect of warming on prey life history, e.g., by reducing the growth rate in prey[45,46]. This makes the interaction of temperature and predator cues especially important in predicting the final outcome of the predator–prey interactions, hence also of SMPE.

Here, we studied combined consumptive (intraspecific SMPE) and non-consumptive (cues of perch, a top predator in ponds) predator effects on life history and physiology in the cannibalistic damselfly *Ischnura elegans* (an intermediate predator in ponds). By crossing consumptive and non-consumptive predator effects with two thermal conditions simulating the current and the predicted increased temperature by 2100 we could assess how both predator-induced effects may change under future warming. We examined SMPE in larval and adult life history and physiological traits and whether non-consumptive top predator effects in combination with increased temperature experienced during damselfly egg and larval stages affect SMPE. We had following set of hypotheses. (1) *I. elegans* shows SMPE in life history and physiological traits. We expected early hatchlings to have advantage over late hatchlings in terms of food acquisition, including cannibalism, leading early hatchlings to show higher values for life history traits (survival, development rate and mass at emergence) and physiological traits (increased energy storage in fat and proteins, and an increased investment in immune function measured as phenoloxidase activity)[4,9,47–50]. (2) SMPE is more pronounced or more likely at the higher temperature as this leads to increased food acquisition through increased activity[51–54], and more/earlier cannibalism[22], but see[55]. (3) SMPE in life history and physiological traits is less pronounced when larvae experience additional stress imposed by top predator cues. *Ischnura* species show reduced activity and metabolic rate in the presence of fish predator cues[56,57], which in turn might cause reduced food acquisition, including cannibalism, and decreased intensity of SMPE[58,59].

Materials and methods

Study species and collection. In this experiment we used the damselfly *I. elegans* as focal species. As top predator, we used the European perch (*Perca fluviatilis*) to impose non-consumptive predator effects on the damselfly. *I. elegans* a common insect species in Europe, occurring from northern Spain to central Sweden[60]. Central Europe populations are uni- and bivoltine (one or two generations per year, respectively), depending on the thermal conditions[61]. Larvae hatch 2–3 weeks after egg laying. Eggs and larval stages commonly share habitats with predatory fish[62]. Fish cues can affect egg and larval life histories and physiology in the study species[63,64].

Adult *I. elegans* females were collected at a pond in Zabierzów Bocheński, Poland (50°03′16.3″N, 20°19′45.7″E). This fish pond contains *P. fluviatilis*. In total, 40 and 36 females were caught in copula on 22 June 2019 (i.e., early group) and on 7 July 2019 (i.e., late group). Females were individually placed in plastic cups with perforated lids and wet filter paper for egg laying, and transported by car in a Styrofoam box to the Institute of Nature Conservation PAS (INC PAS), Krakow, Poland. Adult females were kept in a room at a temperature of 24 °C and natural daylight (photoperiod). Females laid eggs within three days after they had been field-collected. In total 22 clutches were used for the early group treatment, and 26 clutches for the late group treatment. After egg laying, females were released in their natural population.

Ten *P. fluviatilis* (age: 1+) were caught in Dobczyce lake (49°52′27″N, 20°2′55″E) on 19 June 2019. Five fish were used in the experiment, another five were used as a backup. Fish collection and housing were done with a permission from the Local Ethical Committee (ref. 261/2019). Fish were fed frozen *Chironomidae* larvae daily. Fish were not fed with live damselfly larvae, and this to eliminate alarm cues released by larvae exposed to predation[65].

Housing. Egg clutches from early collected females were pooled, and the same was done with eggs from late collected females. The two hatching phenology groups, early (E) and late (L), had 16 days difference in hatching dates, corresponding to the time interval between adult female field collection dates. Such difference in hatching dates occurs in the natural populations because of the long *I. elegans* mating season and mixed voltinism in the sampling region[61,66]. We also created mixed phenology groups, where early hatched individuals shared the same container with late hatched individuals. Note that for the statistical analyses early and late hatched individuals in mixed phenology groups were considered as two different groups, E + L and L + E, where the group E + L referred to the early larvae in the presence of late larvae, and the group L + E to the late larvae in the presence of early larvae. This resulted in four phenology groups: non-mixed E and L, and mixed E + L and L + E. In the non-mixed phenology groups sets of 16 larvae of the same phenology group (E or L) were placed in separate containers, and in mixed phenology groups 8 larvae from E and 8 larvae from L phenology groups were placed in the same container, creating E + L and L + E phenology groups. This way all containers contained 16 larvae. This represents potential scenario for priority effects occurring in nature when comparing densities of unsynchronized hatchlings, compared to otherwise synchronised groups[27]. Each phenology group was studied under the four combinations of two top predator treatments (fish predator cues present and absent) and two temperature treatments (22 °C and 26 °C, hereafter, low and high temperature). Therefore, the set of pooled clutches of each phenology group were separated in four subsets, each subset being assigned to one predator-by-temperature
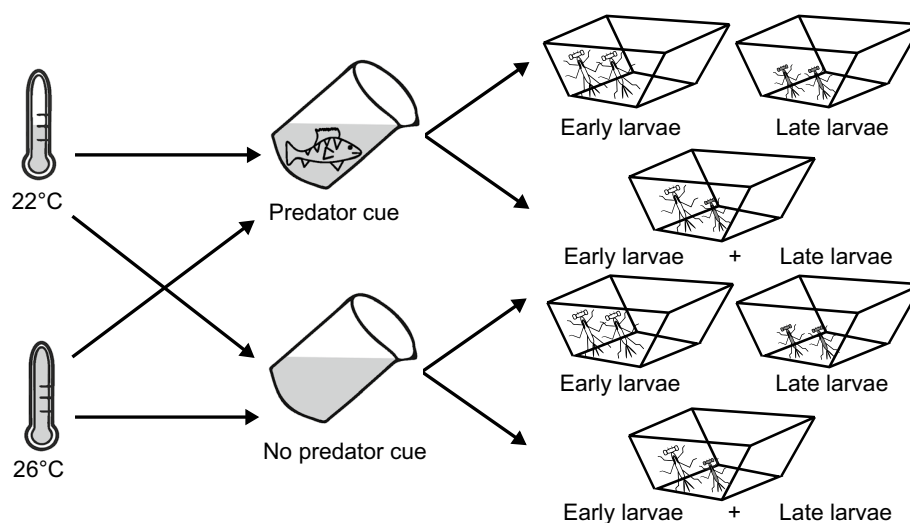
Figure 1. A schematic graph visualizing the full factorial experimental design with two temperature treatment groups crossed with two different predator cue treatment groups, that were each divided into four different phenology groups, resulting in 16 treatment combination groups. Note that in reality 16 larvae were present per container.

treatment combination. This was done before hatching by cutting paper filters on which eggs had been laid and transferring these to separate containers. The temperature treatment started at hatching. The low temperature treatment was based on average temperatures in shallow ponds[67], while the high temperature treatment matched the predicted mean temperature increase by 2100 under IPCC scenario RCP 8.5[68]. This created the following full factorial crossed design: 3 phenology groups × 2 predator cue treatments × 2 temperature treatments × 12 replicated containers × 16 larvae = 2304 individuals at the start (Fig. 1). Throughout the experiment we used a constant photoperiod of L:D 16:8 h, which corresponds to the summer photoperiod, i.e., peak of the larval growth season, at the collection site. We used two climate incubators (Pol-Eko ST 700) for damselfly rearing.

Hatching took place on 6 July 2020 (E group) and 22 July 2020 (L group) at the high temperature, and on 11 July 2020 (E group) and 27 July 2020 (L group) at the low temperature treatment. At hatching, we randomly chose 16 larvae from E and L groups and transferred them to separate containers (16 × 12 cm, height 8 cm) filled with 600 mL of dechlorinated tap water and two nylon net strips, providing hiding space for larvae and climbing structure during emergence. In E + L and L + E groups, we randomly choose 8 larvae from the E group and 16 days later added 8 larvae from the L group. Larvae were fed twice a day (morning and afternoon feeding) with *Artemia salina* nauplii. During the feeding, E and L groups received 10 portions/container (mean = 201.9 nauplii/portion, SD = 17.2). In mixed groups, early hatched larvae received five portions until late hatched larvae were introduced to the same containers. From this time, mixed phenology groups received 10 portions/container.

Every other day, 150 mL of water in every container was refilled with water containing predator cues or no predator cues. Earlier studies have shown that chemical cues of aquatic predators have an average half-life degradation time of ca. 36.5 h[69]. Previous experiments on non-consumptive predator effects in damselfly larvae supported this[33,64].

To distinguish early from late hatched individuals in E + L and L + E groups, we cut the tibia of either one left or one right middle leg. Individuals from E and L groups were marked the same way. The larvae were marked when 30 days old. At that moment, individuals from the two hatching phenology groups could be easily distinguished by size. A preliminary study showed that a 15–20 days difference in hatching dates is sufficient for *I. elegans* larvae to complete two moults regardless of temperature and predator cue treatment (unpublished data). This marking persists until emergence and does not impact the measured traits[9,70].

Freshly emerged individuals were individually transferred to a dry plastic cup and kept for 24 h until the cuticle hardened. Next, damselflies were weighted and frozen at −80 °C for physiology analyses. The experiment ended when the last damselfly larvae emerged.

Response variables. *Life history.* The survival was noted daily between hatching and emergence. Individuals that emerged with fully developed body and wing parts were considered to have emerged successfully. Temperate damselflies are highly cannibalistic and cannibalism increases with increasing body size differences[71]. Based on previous studies on *I. elegans* with larvae reared in groups[25] or individually[64] and on another damselfly species, *Lestes sponsa*, where intrinsic mortality versus cannibalism was determined[29], we assumed that intrinsic mortality was less likely to occur than mortality caused by cannibalism. Larval development time was measured as the number of days between hatching and emergence. One day after emergence, damselfly wet mass was measured to the nearest 0.1 mg with the use of an electronic balance (Radwag AS.62). The growth rate was calculated as adult wet mass divided by the number of days between hatching and emergence. Here, we did not

correct for the hatchling mass because we assumed that hatchling mass did not affect adult mass, as shown in[72]. Also, early handling of hatchlings might interfere with results and larval survival in odonates[73].

Physiology. For physiological analyses, damselfly bodies without legs and wings were grinded with phosphate buffer solution (15 μL for each milligram of wet mass) and centrifuged at 10,000 g for 5 min at 4 °C. All physiology analyses were done on homogenates.

The classical procedure for measuring total body fat in insects[74] was optimized for damselfly bodies. A volume of 8 μL homogenate was mixed with 56 μL 100% sulfuric acid, and heated for 20 min at 150 °C. After cooling down, 64 μL Milli-Q-Water was added. Of this mixture, 30 μL was put in a well of a 384-well microliter plate, and absorbance was measured at 340 nm. The measurements were made on an Infinite M2000 (TECAN) plate reader. To convert absorbances into fat contents, the standard curve of glyceryl tripalmitate was used. The average of three technical replicates per sample was used in the statistical analyses.

Protein content (μg of protein/mg of body mass) was determined using the Bradford[75] method. Of the homogenate, 1μL was mixed with 160 μL of Milli-Q-Water and 40 μL of Bio-Rad Protein Dye. After five minutes of incubation at 25 °C, the absorbance was measured at 595 nm and converted into protein contents using standard curves of bovine serum albumin. The measurements were repeated three times per sample, and the average values used for statistical analyses.

A modified version of the assay described in[76] was used for determining PO activity. Of the homogenate, 10 μL was mixed with 10 μL of phosphoric buffered saline and 5 μL of chymotrypsin. The mixture was put in wells of a 384-well microtiter plate. Afterwards, the samples were incubated for 5 min at room temperature. After incubation, the substrate L-DOPA (1.966 mg dihydroxyphenyl-L-alanine per 1 mL of PBS-buffer) was added and mixed with the samples. Immediately afterwards, the linear increase in absorbance was measured at 490 nm every 20 s for 30 min at 30 °C. The PO activity was quantified as the slope of the reaction curve, and the average of two technical replicates was used for statistical analyses.

Statistical methods. All analyses were run using R 4.0.4[77]. Generalized mixed models with a binomial distribution were used to separately analyse the survival and emergence success (*glmer* function in the lme4 package[78]). The other life history traits (development time, wet mass and growth rate) and the physiological traits (PO activity, fat and protein contents) were analysed using linear mixed models (*lmer* function in the lme4 package[78]). In all models, phenology group, top predator cue treatment, temperature treatment and sex were entered as fixed effects. Initially, models with all possible interactions were run. Interaction terms with $p > 0.1$ were removed from the final models. In all models, container nested within phenology groups were used as random variable. SMPE would be indicated by the pattern where the trait value in the E + L group would be statistically higher (survival, mass, growth rate, fat content, protein content and PO activity) or lower (development time) than in the other phenology groups, and this because of a competitive advantage of early hatched individuals over late hatched ones within mixed-phenology group. If a factor with more than two levels or any interaction term was found statistically significant, post hoc Tukey HSD tests (function *lsmeans*) were run to test pairwise between-level differences. Because of low number of surviving larvae in the L + E group, this group was excluded from all analyses, except for survival until emergence and emergence success.

Ethics declaration. All animal experiments were approved by First Local Ethical Committee for Animal Experiments in Krakow, Poland, and conducted according to Committee guidelines and regulations, reference number 261/2019.

ARRIVE declaration. Manuscript confirming our study has been reported in accordance with ARRIVE guidelines.

Results

Life history. There was a SMPE in survival until emergence, with other factors (temperature, predator cues and sex) not affecting the magnitude of SMPE in this trait. Phenology affected survival until emergence (Fig. 2A, Table 1), with larvae from the E + L group showing the highest survival, and larvae from the L + E group showing the lowest survival (Fig. 2A). None of the interaction terms were significant (Fig. 2A, Table 1). The average percentage of survival in E + L, E, L and L + E groups were 20.1%, 11.98%, 10.29% and 2.1%, respectively (percentages based on raw data). The pattern in SMPE in emergence success was the same as for survival until emergence (Fig. S1, Table 1). There was no SMPE pattern in the other life history traits (development time, adult mass and growth rate).

The phenology treatment had a significant effect on development time (Table 1), with larvae from E and E + L groups taking shorter time for development than larvae from the L group (Fig. 2B). Development time was shorter at the high than at the low temperature (Fig. 2B). Predator cues and sex had no effect on development time (Fig. 2B. Table 1).

The phenology treatment did not affect adult mass (Fig. 2C, Table 1). Larvae reared at the low temperature emerged at a higher mass than larvae reared at the high temperature (Fig. 2C, Table 1). Predator cues increased the temperature effect on mass, further decreasing mass in high temperature and increasing mass in low temperature (temperature × predator cue interaction, Fig. 2 and Fig. S2). Females had a higher mass than males (Tables 1, Fig. S3).

The phenology affected the growth rate (Fig. 2D, Table 1). Larvae from E and E + L groups had higher growth rates than larvae from the L group (Fig. 2D, Tables 1). Larvae grew faster at the high temperature (Fig. 2D,
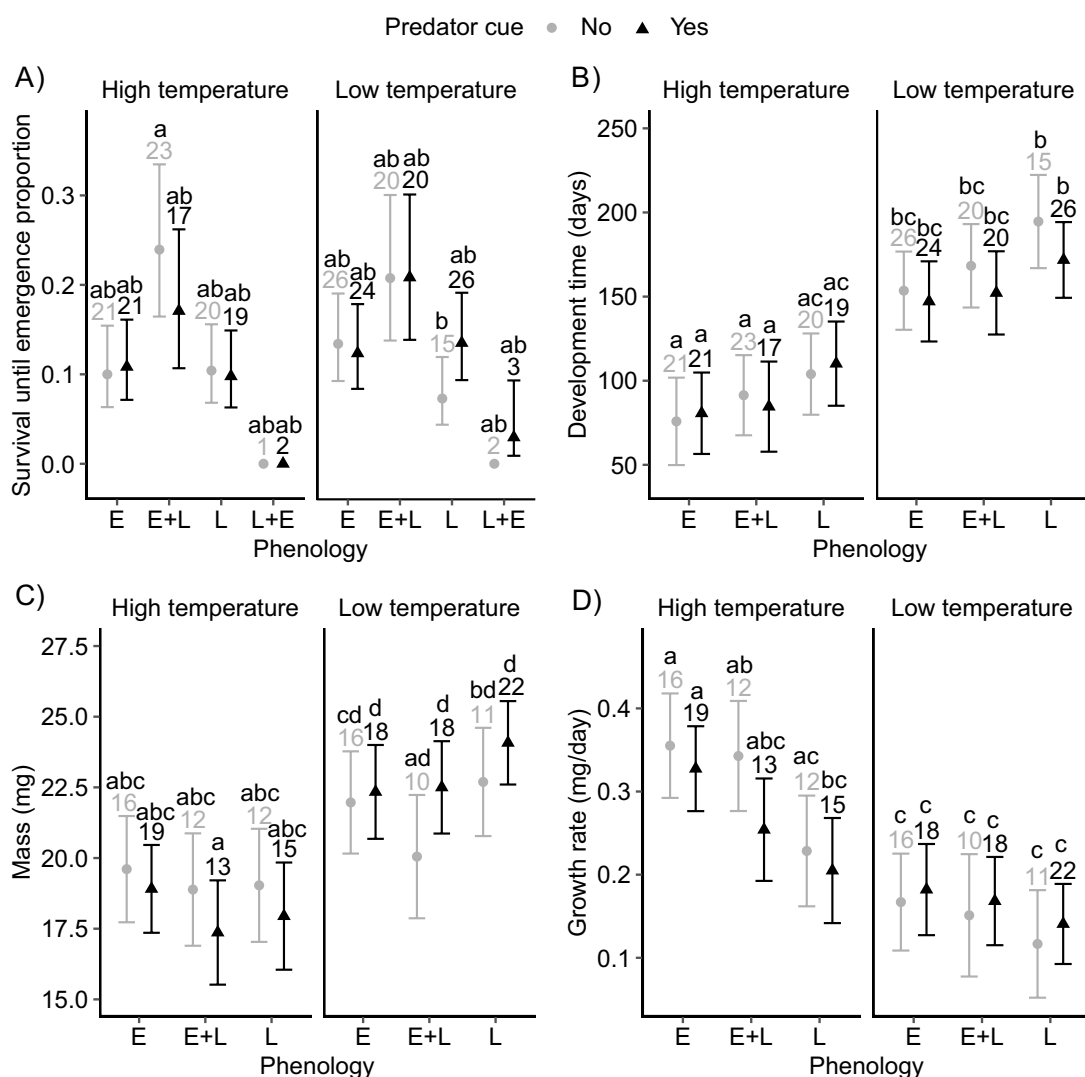
Figure 2. (**A**) Proportion of survival, (**B**) development time, (**C**) adult mass and (**D**) growth rate across different phenology groups (E, E + L, L and L + E), temperatures (high and low) and top predator cues (no and yes). Error bars indicate estimated 95% CI. The numbers on top of error bars represent the number of larvae within each group. E = early larvae, E + L = early larvae in mixed phenology group, L = late larvae, L + E = late larvae in mixed phenology group. All phenological groups are presented, but groups with N < 2 have error bars removed from the plots for clarity. Because of low sample sizes in the L + E phenology group, the L + E group was removed from all analyses, except for analysis on survival rate and emergence success. Letter codes indicate significant differences between phenology groups across both temperatures and predator cue treatments.

Tables 1). Predator cues tended to decrease the effect of temperature on growth rate (temperature × predator cue interaction, P = 0.08, Fig. 2D, Fig. S4, Table 1). Sexes did not differ in growth rate (Table 1).

Physiological traits. There were no SMPE patterns in phenoloxidase (PO) activity and fat content, and a trend for SMPE in protein content. None of the factors affected PO activity, fat content and protein content (Fig. 3A–C, Table 1). However, two interaction terms for protein content showed a trend. The high temperature tended to increase the phenology effect, with the E + L group reared at the high temperature having the highest protein content (phenology × temperature interaction, Figs. 3C, S5, Table 1), indicating SMPE at the high temperature. Males in the L group had a higher protein content than females, while the opposite pattern was present in the E group (phenology × sex, Fig . S6, Table 1). These interaction terms were not supported by the post-hoc tests.

Discussion

We found a SMPE for two life history traits (survival until emergence and emergence success) that was similar across both temperatures supporting the first hypothesis, but not the second hypothesis. In addition, we detected a trend for SMPE for one physiological trait (protein content) but only at the high temperature matching our

Predictor	Df	χ^2	p
Survival until emergence			
Phenology	3	55.59	**< 0.001**
Temperature	1	0.54	0.46
Sex	1	0.04	0.84
Predator cues	1	0.11	0.73
Emergence success			
Phenology	3	46.95	**< 0.001**
Temperature	1	0.17	0.68
Sex	1	0.08	0.78
Predator cues	1	0.47	0.49
Development time			
Phenology	2	11.97	**0.003**
Temperature	1	110.24	**< 0.001**
Sex	1	2.14	0.14
Predator cues	1	0.64	0.42
Mass at emergence			
Phenology	2	3.73	0.15
Temperature	1	53.82	**< 0.001**
Sex	1	16.26	**< 0.001**
Predator cues	1	0.01	0.91
Temperature × predator cues	2	7.75	**0.02**
Growth rate			
Phenology	2	22.79	**< 0.001**
Temperature	1	63.1	**< 0.001**
Sex	1	0.72	0.4
Predator cues	1	0.78	0.38
Temperature × predator cues	2	5.12	0.08
Phenoloxidase activity			
Phenology	2	1.094	0.579
Temperature	1	1.434	0.231
Sex	1	0.01	0.922
Predator cues	1	0.913	0.339
Fat content			
Phenology	2	0.093	0.955
Temperature	1	0.796	0.231
Sex	1	0.244	0.621
Predator cues	1	2.302	0.373
Protein content			
Phenology	2	1.444	0.486
Temperature	1	0.796	0.654
Sex	1	0.786	0.375
Predator cues	1	0.097	0.755
Phenology × temperature	2	5.221	0.074
Phenology × sex	2	4.943	0.084

Table 1. Results from mixed models on life history and physiological traits. Final models included all fixed effects and interactions with p-values < 0.1, whereby p-values ≤ 0.05 were considered significant. Except for the analysis of survival until emergence and emergence success, the L + E phenology group was excluded from analyses due to low sample size (N ≤ 2).

second hypothesis. Expression of other life history traits were in most cases affected by warming and predator cues, but for these traits we did not find SMPEs. The other physiological traits that we quantified showed fixed expressions across treatments, indicating that life history and physiological traits were to some extent decoupled in the study system.

Consistent with SMPE patterns, survival and emergence success were highest in larvae in the E + L group and lowest in those of the L + E group. These results support previous ones[9,18,29,79,80] and confirm that intraspecific competition, including cannibalism, benefits larger individuals. In addition, early-hatched larvae in mixed
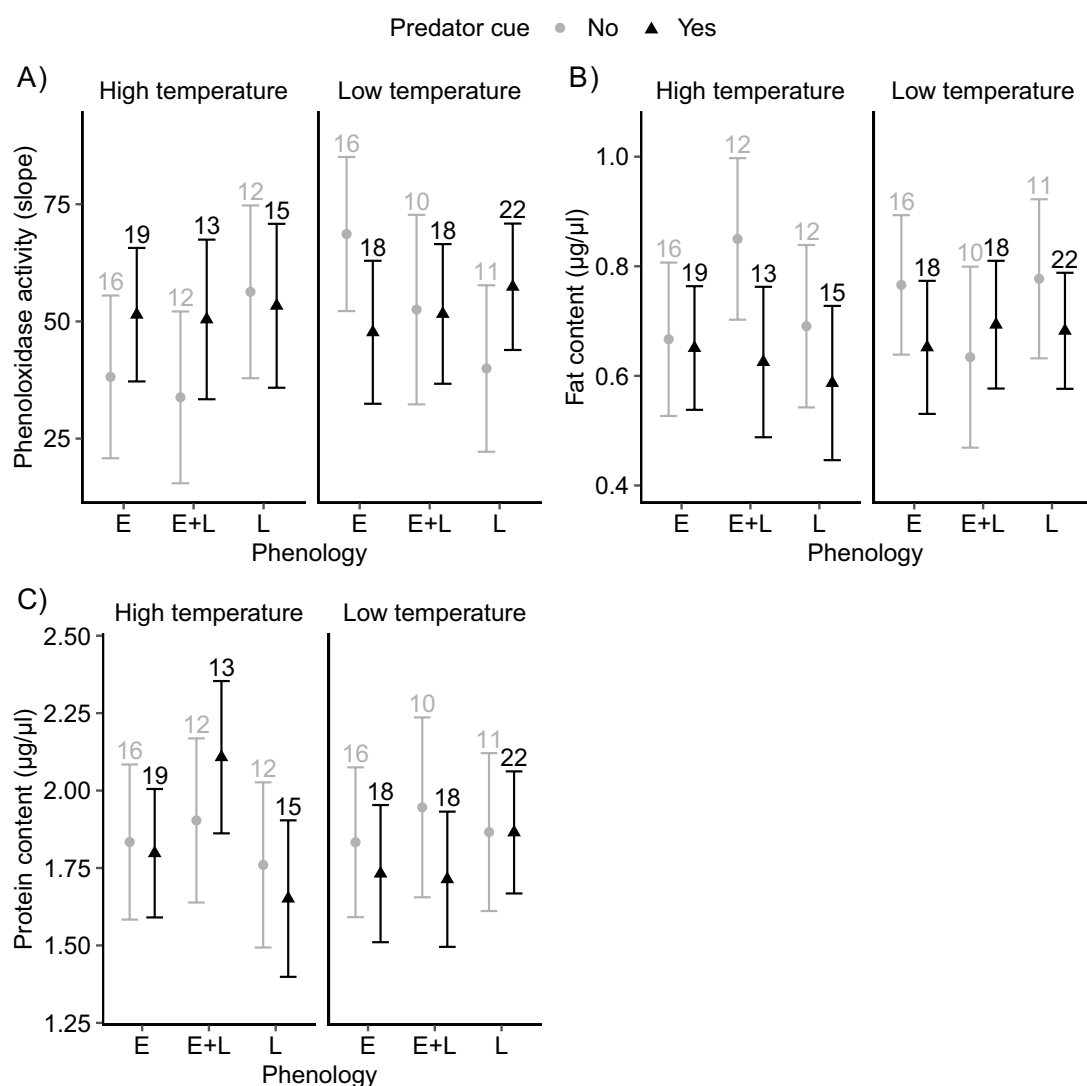
Figure 3. (**A**) Phenoloxidase activity, (**B**) fat content and (**C**) protein content, across different phenology groups (E, E + L and L), temperatures (high and low) and predator cues (no and yes). Error bars indicate estimated 95% CI. The numbers on top of error bars represent the number of larvae within each group. Letter codes were not added due to lack of support of statistically significant differences between groups from post-hoc tests. Abbreviations as in Fig. 2.

phenology groups may have benefited from an increased accessibility to food due to a reduced number of competitors in the containers, i.e. thinning effects[81]. Contrary to the second and third predictions, neither temperature nor predator cues affected the strength of SMPEs in survival and emergence success. We suggest that the impact of these two factors were offset by antagonistic larval interactions. Antagonistic interactions can change larval behavior to avoid predation[32]. Previous studies showed that life history traits in damselflies are altered by non-consumptive predator stress[33,53,64,82–84] and temperature[53,85,86]. But in these studies, the focus was on predator stress on the egg stage or on individually reared larvae, thereby precluding cannibalism. Our current results add that SMPEs in key life history traits can affect population size, but that the strength of SMPEs is weakly altered by other environmental factors.

Despite the highest survival until successful emergence of E + L individuals, we did not detect SMPE in development rate. This result does not support previous results in other ectotherms, including the damselfly *L. sponsa*, which showed SMPE in development time as well as in other life history traits[9,12,87]. In the case of *L. sponsa*, early larvae from mixed groups had the shortest development times[9]. Therefore, we suggest that the differences could be caused by different life cycle characteristics. *L. sponsa* overwinters in the egg stage and is a strictly univoltine damselfly, while *I. elegans* overwinters in the larval stage and has a variable voltinism—with uni- and bivoltine life cycles in the study region[61,66]. These life cycle characteristics influence larval behavior, life history and physiology[88]. As species under high time constraints usually grow and develop faster[89,90], a strictly univoltine species, *L. sponsa*, due to its short larval period after wintering in the egg stage is under higher pressure for rapid growth compared to the univoltine *I. elegans*, which spends winter in the larval stage. However,

a fraction of the *I. elegans* population may complete a second generation within the season (this as a result of cohort splitting, resulting in univoltine and bivoltine fractions), hence proceeds for direct larval development and emergence with no overwintering stage, and therefore is likely more time constrained (but still less than egg-overwintering *L. sponsa*), than the larval overwintering univoltine fraction[88]. Hence, the bivoltine fraction is likely more prone for SMPE. In the experiment, all phenology groups reared at the high temperature finished their larval development and emerged within 100 days (Fig. 2B). This fits the time necessary for the bivoltine fraction to finish its second generation within a season, especially in high temperature conditions, as recorded in local populations of *I. elegans*[66,91]. More studies, preferably in (semi)natural thermo-photoperiod conditions, allowing larval direct development until emergence (bivoltine) and larval overwintering (univoltine) are needed to clarify the effect of within population variation in voltinism on SMPE in damselflies.

Early hatched *I. elegans* from both mixed- and non-mixed phenology groups had shorter development times than late hatchlings. Shorter development times in early hatchlings were accompanied with elevated growth rates, and this led early hatchlings to reach similar mass at emergence as late hatchlings. Hence, there was apparently no trade-off between age and mass at emergence, which is often reported in ectotherms[92–96], but see[97]. These plastic life history responses of early hatchlings may be adaptive. Early emerged individuals mature early in the season and have higher mating success than delayed ones[98]. Usually there is also a positive association between adult mass and components of mating success[99,100]. Additionally, we did not detect mortality costs of fast development rate, which is often reported[101–103]. Early emergence is likely adaptive by allowing the completion of an extra generation within a year, i.e. bivoltinism, especially when temperature conditions are permissive[61,66,104]. However, selection for early emergence is probably relaxed because of highly unsynchronized mating over the flight season in *I. elegans*[105]. Contrary, in damselflies with synchronized, early season emergence and mating such as *Coenagrion armatum*[106], *Coenagrion hastulatum* or *Coenagrion puella*[107] selection for early date emergence, fast development and early maturation is likely strong. Finally, maternal effects could have also played a role in shaping the faster development of early vs late offspring. It has been reported that when mothers age, their condition may drop and the offspring quality decrease[108–110]. A higher quality of early hatchers could positively affect their development rate and decrease age at emergence with no trade-off between these two traits. The few studies that focused on maternal effects in damselflies reported weak or no impacts of the mother on her offspring quality[72,111,112]. In the current study we could not determine if maternal effects had an impact on life history and physiology traits, but it is worth investigating in the future.

We found ecologically important temperature effects on life history traits which did not show SMPEs. As expected, the high temperature decreased development time, and the shorter development time resulted in a lower mass at emergence. This elevated temperature-driven trade-off was somewhat reduced by increased growth rates at the high temperature, yet, the increase of growth rate was not strong enough to fully compensate the shorter development time. A similar incomplete compensating mechanism under warming was shown in previous studies, including studies on damselflies[113] and is considered one major mechanism for the here observed temperature-size rule where animals get smaller at higher temperatures[114].

It has been demonstrated that non-consumptive predator effects can change prey life history traits[32,115,116], and could therefore potentially weaken or remove SMPE in prey, by, for example, reduced foraging rate in prey due to predator avoidance[117–119]. Here, we show that predator cues affected damselfly life history, but without having an effect on SMPEs. Specifically, predator cues reduced larval growth rate, leading to a lower mass at emergence, but only in the high temperature treatment (predator cue × temperature interaction for growth and mass). This suggests that the expected temperature rise will likely increase non-consumptive predator stress in *I. elegans*, with potentially negative fitness consequences. Similar results were shown in previous studies on other ectotherms[45,46], including a damselfly[120]. These results could be explained as follows: predator stress increases physiological stress in prey, causing more energy to be allocated to costly defence mechanism rather than growth rate[121]. Taken together, current and previous results indicate that warming temperature may magnify the effects of predator-induced stress in prey, but that the increased predation stress may not affect SMPEs in prey.

The increased temperature lead to a weak SMPE in protein content (phenology × temperature interaction, $p = 0.074$), a fundamental component of various body structures, including muscles[122], whereby the early larvae in the mixed group had a higher protein content under warming. This matched our second prediction of SMPEs being stronger or more likely at the high temperature. SMPE may be more likely under warming because a higher metabolism allows faster and more pronounced reactions to interactions between organisms, as well as the latter being stronger in general. In cannibalistic species, increased interactions result in higher cannibalism rates[7,123]. As conspecifics represent a rich source of proteins for cannibals[124], increased cannibalism may lead to a higher protein content which can have positive effects on body condition during the larval stage[125,126]. As proteins make up an important part of the swim muscles in damselfly larvae, it may contribute to a better predator escape performance. Furthermore, this may generate positive carry-over effects across metamorphosis in the adult stage. For instance, proteins play an important role in ensuring proper wing elasticity, and as building blocks of flight muscles and the exoskeleton[127–129]. Intriguingly, the increased protein content under warming was not traded off against a faster growth rate, as it happened in body mass. This suggests that the larvae invest more energy into proteins than into other traits shaping final body size. It would be interesting to study in detail into which tissues the early hatched individuals invested more in the context of SMPEs.

We did not detect SMPEs in immune function (PO activity) and energy storage (fat content). These traits had similar values across all experimental treatments, suggesting fixed responses. These results are surprising because previous studies showed that PO activity and fat storage increased under warming, and decrease under predator pressure but, again, when larvae were reared individually[50,130]. That the physiological traits did not follow the SMPE hypothesis confirms previous results in *L. sponsa*[29]. Yet, in the latter species trait values showed plastic responses when individuals were exposed to time stressed conditions: PO activity decreased and fat content

increased[29]. In the current experiment we did not impose time stress, but it would be interesting to study this stress on SMPE in *I. elegans* and link it with variable voltinism in this damselfly.

In summary, our results confirm that SMPEs caused by differences in hatching phenology are an important factor that by shaping survival and emergence success can promote early emergence of amphibious and cannibalistic organisms in a population. Other central findings of current study were that warming and non-consumptive effects imposed by a top predator did not affect SMPE for life history traits, yet warming did generate a weak SMPE for larval protein content that may adaptively carry over to the adult stage. In agreement with theory[20], and current results, we suggest that given the high tendency for larval cannibalism, SMPEs in *I. elegans* could lead to directional selection for early adult breeding.

Data availability

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Competing interests

The authors declare no competing interests.

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PAPER 4

Phenological shifts in a warming world affect physiology and life history in a damselfly

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Article

Phenological Shifts in a Warming World Affect Physiology and Life History in a Damselfly

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Simple Summary: Climate warming affects phenological events of cold-blooded organisms. In this analysis we studied, in laboratory conditions, the impact of warming and hatching dates on key life history and physiological traits in a cannibalistic damselfly, *Ischnura elegans*. Larvae were reared in groups from hatching to emergence through one or two growth seasons, depending on the voltinism. Larvae were equally divided by hatching dates (early and late) and temperature treatment (current and warming). Early and late hatched groups were not mixed. Despite no difference in cannibalism rate between different hatching dates and temperatures, early hatched larvae reared under warming had elevated immune function measured as phenoloxidase (PO) activity. This increased PO activity was not traded off with life history traits. Instead, age and mass at emergence, and growth rate were mainly affected by temperature and voltinism. Our results confirm the importance of phenological shifts in a warming world for shaping physiology and life history in a freshwater insect.

Abstract: Under climate warming, temperate ectotherms are expected to hatch earlier and grow faster, increase the number of generations per season, i.e., voltinism. Here, we studied, under laboratory conditions, the impact of artificial warming and manipulated hatching dates on life history (voltinism, age and mass at emergence and growth rate) and physiological traits (phenoloxidase (PO) activity at emergence, as an indicator of investment in immune function) and larval survival rate in high-latitude populations of the damselfly *Ischnura elegans*. Larvae were divided into four groups based on crossing two treatments: early versus late hatching dates and warmer versus control rearing temperature. Damselflies were reared in groups over the course of one (univoltine) or two (semivoltine) growth seasons, depending on the voltinism. Warming temperature did not affect survival rate. However, warming increased the number of univoltine larvae compared to semivoltine larvae. There was no effect of hatching phenology on voltinism. Early hatched larvae reared under warming had elevated PO activity, regardless of their voltinism, indicating increased investment in immune function against pathogens. Increased PO activity was not associated with effects on age or mass at emergence or growth rate. Instead, life history traits were mainly affected by temperature and voltinism. Warming decreased development time and increased growth rate in univoltine females, yet decreased growth rate in univoltine males. This indicates a stronger direct impact of warming and voltinism compared to impacts of hatching phenology on life history traits. The results strengthen the evidence that phenological shifts in a warming world may affect physiology and life history in freshwater insects.

Keywords: phenology; life history; immune function; freshwater insect; *Ischnura elegans*; voltinism; climate change

1. Introduction

Climate change affects organisms' phenology, i.e., periodic life history events influenced by seasonal and interannual variations in climate [1,2]. Climate change might thus

indirectly affect ecological interactions because changes in temperature affect arrival of migrants, egg hatching and other phenological events that may alter competition between organisms [3,4]. Other indirect effects of rising temperatures are increased competition rates, including cannibalism, which can influence population dynamics [5,6]. These changes in organisms' interactions are expected to happen as species and populations show genetic variation and phenotypic plasticity in phenological events [7–9]. At a more mechanistic level, the timing of phenological events and temperature may affect physiology [10,11]. Therefore, variation and plasticity in physiological parameters should be considered when predicting organisms' responses to global change.

An important type of organismal response to warming is the expression of different developmental trajectories. Alternative developmental trajectories can lead to different ages of sexual maturation. In organisms with complex life cycles such as insects, different developmental trajectories can, in the extreme case, result in cohort splitting [12]. Cohort split occurs when organisms that start their development at the same time follow different, genetically determined physiological pathways that result in different durations of the larval stage. Such cohort splitting was reported in a temperate population of the damselfly, *Calopteryx splendens*, that is, while the predominant fraction of the population completed larval development within one year (univoltine), small fractions took either half a year (bivoltine) or two years (semivoltine) to complete development [13]. In insects, cohort splitting is an important factor in shaping fitness traits such as emergence date, adult body mass and size, and mating success [14]. Cohort splitting might also affect intraspecific competition, especially due to differences in body size, with the slower growing cohort expected to perform worse than the faster growing cohort, and hence reach maturation later [15,16]. In addition, because fast growth leads to earlier maturation and mating, a fast cohort can increase the number of generations per year, and hence fitness components [17,18]. For example, multivoltine (more than two generations per year) populations of the butterfly, *Lycaena hippothoe*, showed higher reproductive success than univoltine populations, and this despite a cost of decreased mass in multivoltine populations [19]. It is, therefore, important to consider cohort splitting when predicting population responses to climate warming because within and between cohort competition for food, space or mating partners can interact with temperature [6,20].

Here, we study how egg hatching phenology and temperature affect juvenile life history, physiology and competition across metamorphosis in high-latitude populations of the damselfly, *Ischnura elegans*. The larvae of damselflies are cannibalistic. The larval cannibalism rate increases when damselflies differ in size [21–23], experience prey scarcity [21,24,25] but see [26] and warming temperature [5]. At high latitudes, damselfly populations can have variable voltinism—between one to three years for completing a generation, i.e., uni-, semi- and partivoltine life cycles [27] (Ulf Norling pers. comm.), with the univoltine being more seasonally time constrained than the semi- and partivoltine, i.e., having a shorter time window available (one vs. two or three growth seasons) for development and growth [28–30]. We made two predictions. First, we predicted that when reared in a group with early hatchers and in a group with late hatchers, early hatchers will show a higher growth rate, higher mass and lower age at emergence (i.e., increased voltinism), higher investment in immune function measured as phenoloxidase (PO) activity and will experience more competition resulting in decreased survival compared to late hatchers. For early hatchers, early hatching provides more time available for larval development within the growth season and opportunity for completing juvenile development during the next growth season [27], resulting in a fast cohort, i.e., univoltinism [31]. Early hatching should therefore increase development rate and PO activity through increased growth efficiency [7,32,33] and competition over prey [34] compared to late hatchers. This is caused by higher activity and foraging rates of time constrained, univoltine larvae [35]. Late hatchers are predicted to postpone emergence until the following season(s), resulting in a slow cohort, i.e., semi- or partivoltinism, with decreased parameters of development and growth rate, mass at emergence and PO activity [31], and increased survival rate [36].

Second, we predict that warming will increase development rate, growth rate and voltinism, and will decrease mass at emergence and survival rate. Higher temperatures increase metabolism and activity in ectotherms, leading to increased feeding, growth rates, and competition [37,38]. Mass at emergence will be reduced because of a temperature-driven trade-off between age and mass at emergence (temperature–size rule) [39,40]. We further expect that warming will promote increased investment in immune function due to thermal dependence of enzymatic precursor of phenoloxidase (immune function protein), pro-phenoloxidase [41], and will potentially increase pathogen presence at higher temperatures [42,43].

2. Materials and Methods

2.1. Study Species and Field Collection

Ischnura elegans is a common damselfly in Europe. The species occurs from central Sweden to northern Spain [44]. *I. elegans* shows a rapid geographic shift towards the north as a result of climate change [45]. This damselfly has a long aquatic larval stage where most of its growth and development occurs, and thereafter a short terrestrial adult stage with dispersal and reproduction. Larvae hatch 2–3 weeks after egg laying. Larva is the overwintering stage [22]. Individuals with a different voltinism overwinter in various instars [46]. High-latitude populations need 1–3 years for completing larval development, i.e., have a uni- and semivoltine life cycle [27] (Ulf Norling pers. comm.). This is longer than in southern populations which can be bi- or multivoltine, i.e., have two or more generations per year, respectively [47].

To collect eggs, adult *I. elegans* females in tandem with a male were collected at two ponds in central Sweden near Uppsala (59°60′ N 17°40′ E and 59°53′ N 17°38′ E). Females were individually placed in plastic cups with perforated lids and wet filter paper for egg laying at room temperature and the natural photoperiod. Oviposition occurred within three days after females had been field-caught. Eggs were collected after three days, and surviving females were released into their natural populations. Eggs for the early hatching treatment were acquired from females caught 27–30 June 2019, and for the late hatching treatment 13–14 July 2019. In total, 33 early-laid clutches (15 and 18 clutches from the first and second pond, respectively) and 22 late-laid clutches (5 and 17 clutches from the first and second pond, respectively) were collected. Egg clutches were transported to the Institute of Nature Conservation PAS in Krakow, Poland. Clutches from different females and ponds were pooled, creating two groups of eggs, early and late.

2.2. Experimental Rearing

The experiment was performed at the Institute of Nature Conservation PAS, Krakow, Poland. *I. elegans* eggs were divided into groups based on their collection dates. Based on date of collection, two hatching phenology groups were created—early (caught in June) and late (caught in July) hatchers. During the first growth season (i.e., the season during in which the eggs were laid), early and late hatched larvae were kept the same temperature (21 °C) and photoperiod (L-D 22–2 h). These conditions reflect natural conditions at the sampling sites at the peak of the growth season, i.e., summer solstice. Temperature treatments were introduced during the following growth season, that is, after the first larval overwintering, marking the start of the experimental treatment. To follow natural temperature changes during the second and third growth seasons, we changed the temperature weekly to follow mean temperature changes in shallow parts of waterbodies (the optimal habitat for damselfly larvae, Corbet 1999) in central Sweden. Temperatures were modelled using the FLake model [8,9,48]. These temperatures are reliable estimates of the field-measured water temperatures in ponds [49]. During the second and third growth seasons, we increased the photoperiod by 2 h, resulting in a L-D 24–0 h (i.e., summer solstice). Based on previous results on temperate damselflies [30], we increased the photoperiod to trigger faster larval growth and development across all treatment groups. Experimental temperatures and photoperiods are shown in Figure 1.
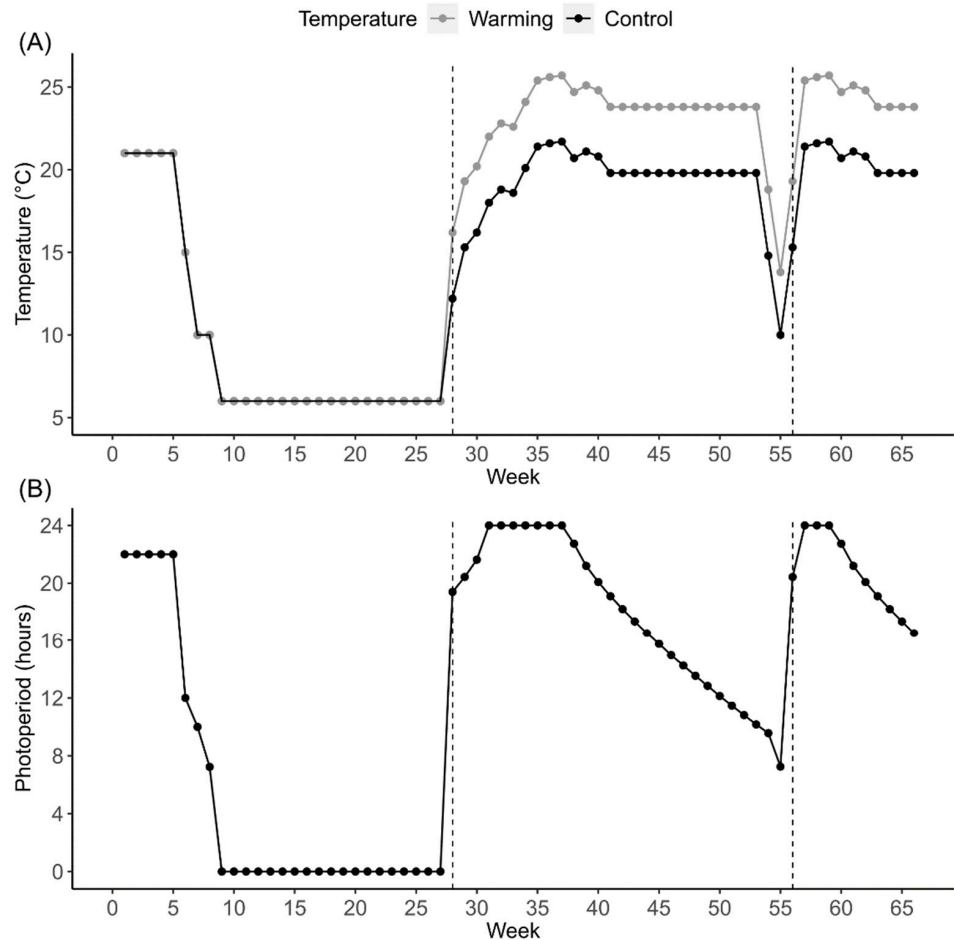
Figure 1. (**A**) Temperatures in °C and (**B**) photoperiods in hours of light during a 24 h cycle used during the experiment. Temperature treatment groups differed by 4 °C. The photoperiod was kept identical in both temperature treatments. The first growth season lasted from week 1 to week 8. The second growth season lasted from week 28 to week 55 (start is indicated by a dashed line to the left). The third growth season lasted from week 56 to week 66 (start is indicated by a dashed line to the right). During weeks 9 to 27 we installed winter conditions at 6 °C and total darkness. The short-term temperature and photoperiod drop at week 55 simulated the second winter condition.

The early and late groups hatched on 16 July and 1 August 2019, respectively. At hatching, clutches of the same date group from the two populations were combined. Next, eight larvae from the same phenology group were placed together in containers (16 cm × 12 cm, height 8 cm) filled with 600 mL of dechlorinated tap water. In these containers the larval density (417 larvae per m^2) was within the range of larval densities observed for *Ischnura* species in nature [50,51]. Two nylon nets were put inside each container as a substitute for submerged vegetation. On 24 August 2019 we started preparing larvae for winter by gradually decreasing temperature and day length. On 12 September 2019 we started winter conditions of 6 °C and total darkness. We kept larvae in these conditions until initiation of the following spring. On 24 January 2020 we started spring conditions at 12.2 °C (control temperature) and 16.2 °C (warming temperature), and for both thermal treatments the photoperiod was L-D 19:24–4:36 h. The difference of 4 °C matches the predicted mean temperature increase by 2100 under IPCC scenario RCP 8.5 [52]. Temperatures and photoperiods were changed at weekly intervals, thereby keeping the 4 °C temperature difference (Figure 1). A second winter period was induced for larvae by short-term temperature and photoperiod drop at week 55. The short time period of the second

winter was used for logistical reasons. One could argue that this may not be sufficient to initiate and end larval diapause, which could affect larval traits measured during the following growth season. However, the emergence distribution after the second wintering shows a peak at week 60 (ca. one month after the second winter period ends, Figure S1). This suggests that the gradual decrease and increase in temperature and photoperiod prior to and after each winter stimulated larvae to enter and then terminate winter diapause. The experiment was concluded when last individual emerged during week 66.

Throughout the experiment, larvae were fed two times per day during the growth seasons and one time every other day during the winter. They received five portions per container of *Artemia salina* nauplii (mean = 201.9 nauplii/portion, SD = 17.2). *A. salina* nauplii are often used as a food source in Odonata research [53,54] due to ease in rearing this food source. Additionally, *I. elegans* are generalists, eating prey appropriately to their occurrence in the field [26]. During the following spring conditions, larvae received eight newly hatched *L. sponsa* as supplementary food. Early and late hatched groups received the supplementary food on 28 January and 10 February 2020, respectively. *L. sponsa* hatchlings originated from eggs acquired from 33 adult females caught at two ponds near Sundsvall, Sweden (62°25′ N 17°16′ E and 62°26′ N 17°21′ E) on 1 August 2019. One day after emergence, adults were weighed and frozen at −80 °C for the physiological analyses.

2.3. Response Variables

Larval survival was recorded daily. Individuals that attempted to emerge (dead larvae on the nylon net above the water surface and live, fully emerged adults on nylon nets) were categorized as surviving until emergence. In this study, intrinsic mortality was not verified, and mortality caused by both cannibalism and intrinsic reasons was used to estimate survivorship. However, in a previous group-rearing experiment with *Lestes sponsa*, intrinsic mortality caused by means other than cannibalism was low and did not differ between treatments [55]. Based on this, we assume mortality in *I. elegans* was mainly caused by cannibalism. Development time (i.e., age at emergence) was measured as the number of days between egg hatching and adult emergence, with the exclusion of the winter period(s) where no development is expected [56]. Adult wet mass (mg) was determined one day after emergence by measuring damselfly weight on an electronic balance (Radwag AS.62, Krakow, Poland). Growth rate was calculated by dividing adult wet mass by larval development time. Individuals were classified to one of the two voltinism groups, depending on the emergence season: those that emerged during the second season between week 19 and 46 were considered univoltine, and those that emerged during the third season between week 47 and 66 were considered semivoltine (Figure 1).

Phenoloxidase (PO) activity was quantified from damselfly bodies whose legs and wings had been removed. The bodies were grinded, mixed in phosphate buffer solution (15 μL for each milligram of wet mass) and centrifuged at 10,000× *g* for 5 min at 4 °C. The assay to measure PO activity was based on Stoks et al. (2006). A mixture of 10 μL of homogenate with 105 μL of phosphoric buffered saline (PBS) and 5 μL of chymotrypsin was incubated for 5 min in a 384-well microtiter plate. Afterwards, L-DOPA (1.966 mg dihydroxyphenyl-L-alanine per 1 mL of PBS-buffer) was added to the samples, followed by immediate measurement of the linear increase in absorbance at 490 nm every 20 s for 30 min at 30 °C. The average values of the slope of the linear part of the reaction curve from two technical replicates was used for the statistics. To correct the PO activity, the protein content of the samples was measured using a modified Bradford [57] procedure. A mixture of 1 μL of homogenate, 160 μL of Milli-Q-water and 40 μL of Bio-Rad Protein Dye was incubated for 5 min at 25 °C. Afterwards, the absorbance at 595 nm was measured and converted into protein content using a standard curve of bovine serum albumin. The averages of three technical replicates per larva were used for statistical analyses. To express the activity of PO per total protein content, the values of slope of the reaction curve for PO was divided by the values of protein content.

2.4. Statistical Analyses

　　To analyze the response variables of survival until emergence and voltinism, generalized mixed models with a binomial error distribution were used. In these models early versus late hatching, control versus warming, and sex were fixed effects. Note that voltinism was not included as an explanatory variable in the survival analysis because survival was estimated at emergence only, and not between growth seasons. Other response variables (age and mass at emergence, growth rate and PO activity) were analyzed using linear mixed models. In these models hatching phenology, temperature, sex and voltinism were included as fixed effects. Container was included as a random effect to account for multiple larvae sharing the same container. At first, models with all possible interactions were performed, then all interactions with $p \geq 0.05$ were removed at once. Final models included all fixed effects and interactions with $p \leq 0.05$. Post-hoc Tukey HSD tests were used to assess pairwise between-level differences. To perform statistical analyses, we used R 4.0.4 software (Krakow, Poland), with packages lme4 [58] for mixed-effect modelling and emmeans [59] for post-hoc tests.

3. Results

　　In total, 113 (12% per total at the start) larvae attempted to emerge, and among these 37 (32.7%) individuals were univoltine and 76 (67.3%) individuals were semivoltine. Out of 92 larvae that emerged with success, 35 (38.0%) were univoltine and 57 (62.0%) were semivoltine (Table S1). The percentage of emergence attempts that were successful per total attempts was 81.4%. More univoltine (94.6%) than semivoltine (75.0%) individuals attempting to emerge did so successfully ($\chi^2 \leq 5.036$, $p \geq 0.025$). Hatching phenology, temperature and sex did not affect survivorship (Figure 2A, Table 1). Early versus late hatching did not affect voltinism (Figure 2B, Table 1). Under warming more univoltine than semivoltine individuals emerged, with the opposite happening at the control temperature (post-hoc contrasts for semivoltine/univoltine ratio, early versus late hatching date and control versus warming temperature: early hatching, control temperature—early hatching, warming temperature $p < 0.01$; late hatching, control temperature—late hatching, warming temperature $p < 0.01$; early hatching, warming temperature, late hatching, control temperature $p < 0.01$, Figure 2B, Table 1). Males showed equal ratio of uni- and semivoltine individuals, whereas females tended to have higher ratio of semivoltine individuals (Figure S2, Table 1). Body mass was not affected by hatching phenology (Figure 2C, Table 1). Temperature and voltinism affected mass in an interactive way: univoltine individuals were heavier than semivoltine individuals, but only at the control temperature. Warming decreased mass, but only in univoltine individuals (interaction temperature × voltinism, Figure 2C and Figure S3, Table 1). Females were heavier than males (Figure S4, Table 1). Early hatched individuals took longer to develop than late hatched ones (Figure 2D and Figure S5, Table 1). Hatching phenology did not affect development and growth rate (Figure 2D,E, Table 1). Temperature, sex and voltinism affected both development time and growth rate in an interactive way. Warming shortened development time, but only in univoltine females (interaction temperature × sex × voltinism, Figure S6, Table 1). Warming in univoltine females increased growth rate, while in univoltine males it decreased growth rate (interaction temperature × sex × voltinism, Figure S7, Table 1).

　　Hatching phenology and temperature affected PO activity in an interactive way. Early hatchers showed increased PO activity, but only in the warming treatment (interaction hatching phenology × temperature, Figure 2F and Figure S8, Table 1). Females had a higher PO activity than males, but only in the warming treatment (interaction temperature × sex, Figure S9, Table 1). PO activity was not affected by voltinism (Figure 2F, Table 1).
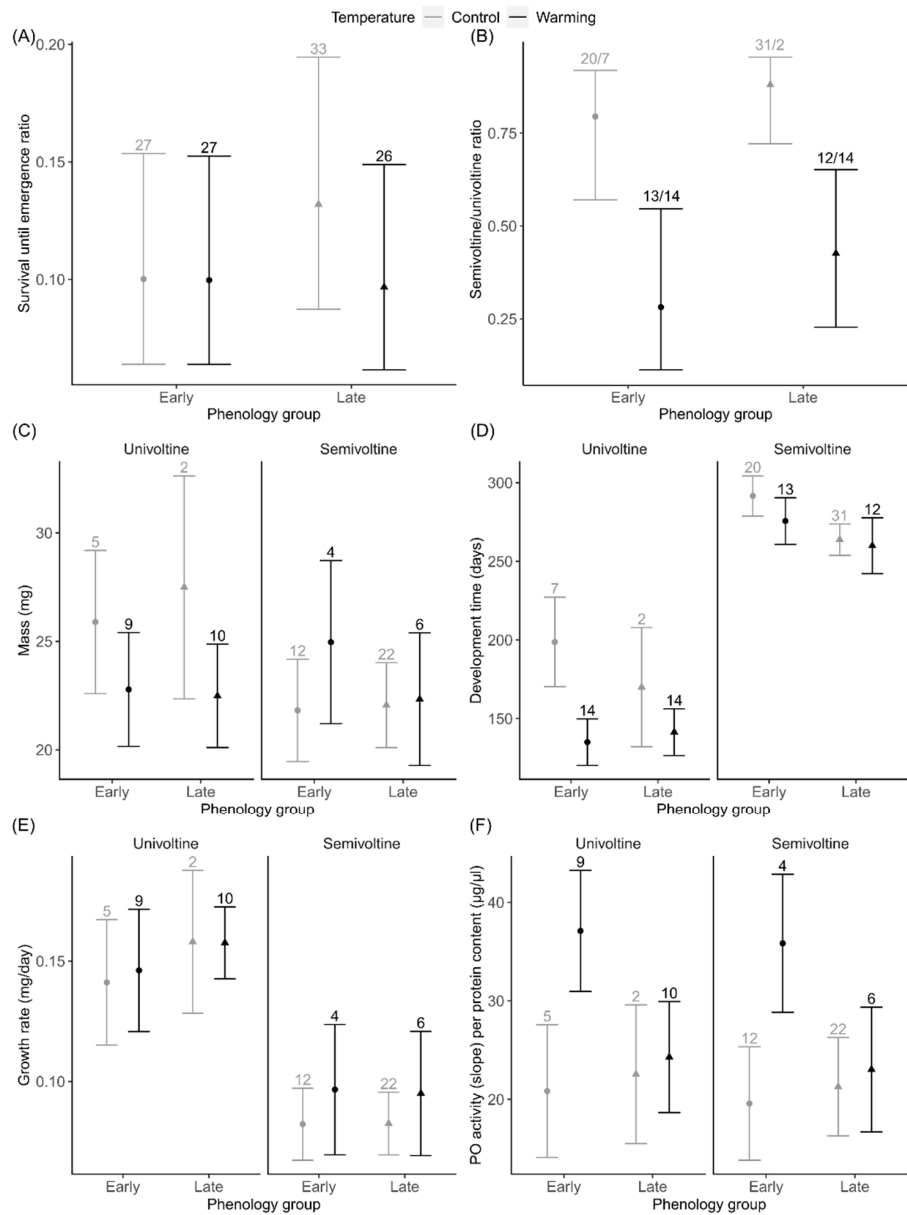
Figure 2. The effects of hatching phenology and warming on the life history and physiology of univoltine and semivoltine *Ischnura elegans*: (**A**) larval survival until emergence (ratio of surviving to all); (**B**) voltinism (ratio of semivoltine to univoltine); (**C**) mass at emergence; (**D**) development time; (**E**) growth rate; (**F**) phenoloxidase (PO) activity. Note that the voltinism effect was excluded from the survival analysis because survival was estimated at emergence only, and not between growth seasons. Error bars indicate estimated 95% CI. The numbers at the top of the error bars represent the number of damselflies within each group.

Table 1. Results from mixed models testing for effects of hatching phenology, temperature, sex and voltinism on *Ischnura elegans* survival, voltinism, mass, development time, growth rate and phenoloxidase (PO) activity across emerged adults. Voltinism was analyzed as a response variable and as one of the explanatory factors when analyzing other traits. Note that the voltinism effect was excluded from the survival analysis because survival was estimated at the emergence only, and not between growth seasons. Final models included all fixed effects and interaction terms with *p*-values ≤ 0.05. *p*-values below 0.05 are in bold.

Predictor	Df	χ^2	*p*
Survival			
Hatching phenology	1	0.379	0.548
Temperature	1	0.583	0.444
Sex	1	0.094	0.760
Voltinism			
Hatching phenology	1	1.078	0.299
Temperature	1	14.235	**<0.001**
Sex	1	3.155	0.076
Mass			
Hatching phenology	1	0.616	0.433
Temperature	1	0.468	0.494
Sex	1	13.651	**<0.001**
Voltinism	1	7.516	**0.006**
Temperature × voltinism	1	7.216	**0.007**
Development time			
Hatching phenology	1	11.437	**<0.001**
Temperature	1	7.526	**0.006**
Sex	1	0.280	0.600
Voltinism	1	385.402	**<0.001**
Temperature × voltinism	1	4.345	**0.037**
Temperature × sex	1	3.720	0.054
Temperature × sex × voltinism	2	6.293	**0.043**
Growth rate			
Hatching phenology	1	0.095	0.758
Temperature	1	0.003	0.954
Sex	1	4.455	**0.035**
Voltinism	1	158.661	**<0.001**
Temperature × sex	1	3.729	0.053
Temperature × sex × voltinism	3	13.344	**0.004**
PO activity			
Hatching phenology	1	4.999	**0.034**
Temperature	1	10.178	**0.001**
Sex	1	2.639	0.105
Voltinism	1	1.630	0.201
Hatching phenology × temperature	1	15.047	**<0.001**
Temperature × sex	1	5.963	**0.015**

4. Discussion

Here we explored how hatching date and temperature affected key life history traits such as survival and age and mass at emergence, and immune function at emergence in a damselfly. Given the predicted climate change scenarios in the future, it is important to understand how shifts in life history events and environmental conditions affect these individual traits. Our results showed a complex interplay between phenology and warming. This suggests that it may not be easy to predict how future climate change will affect population abundance and dynamics.

We predicted that early hatched individuals should have a higher mass at emergence compared to late hatching individuals. We found no support for this prediction, but we found that voltinism and temperature affected this trait. Univoltine damselflies showed a

higher mass than semivoltine individuals, but this difference disappeared under warming. This result is in contrast with the compound interest hypothesis, which states that warming increases the number of generations per season, but with a cost of a lower mass at maturation [19,60,61]. On the other hand, adding an extra season for completing a generation (i.e., here, a switch from uni- to semivoltine life cycle) should lead to a greater mass and/or size at emergence [28], hence increased reproductive success [62,63] but see [64,65]. In our case, the opposite was found. This suggests a two-fold potential fitness benefit for univoltine ischnurids: a shorter generation time and a larger mass at emergence. The effect of mass on fitness might be expressed by greater emergence success per emergence attempt in univoltines compared to semivoltines. A previous study on ischnurids indicated that emergence success was positively correlated with mass in the final larval instar [66], which in turn correlates with other fitness traits in adult insects, e.g., structural size and fecundity [22,67,68].

Interestingly, hatching phenology did not affect voltinism, but warming increased the ratio of univoltine individuals, confirming that warming will increase the number of generations per year in ectotherms, also those living at high latitudes. However, at some point this trend for increased voltinism might become less beneficial, and this is because of a decreased mass in fast developing damselflies [69], resulting in a decreased fitness.

The temperature-dependent difference in mass in univoltine ischnurids supports the temperature–size rule (TSR), where warming leads to acceleration of development rate without relative increase in growth rate, leading to smaller mass and size at maturation [39,70]. Our results on development and growth rates add support for this rule. The above suggests that warming will reduce mass at emergence through increased development rates, as is generally expected in ectothermic organisms [70,71], and that advanced hatching dates will have minor effects on age and mass at emergence. Interestingly, the TSR pattern did not occur in semivoltine individuals. This result supports the compound interest hypothesis, where organisms with decreased voltinism are expected not to react or to react weakly to increasing temperature by changing their development rates and mass at emergence (for example, a weak TSR response in univoltine population of a butterfly *Lycaena hippothoe* compared to multivoltine population [19]).

We found a trend for a positive relationship between age and mass at emergence within the univoltine cohort: the lighter individuals emerged at the beginning of the growth season (a trend for a positive correlation between age and mass at emergence, r = 0.34, p = 0.092, Figure S10). Such positive correlation is rather rare in insects, including odonates [72,73], and the opposite pattern is commonly reported [74–76]. Within and across seasons, temporal variation in mass at emergence is not well understood, and both internal (physiological) and external (ecological, e.g., competition and temperature) factors were suggested to explain the pattern [28,77,78]. In our case, a likely explanation, though not quantified in this study, might be that larvae from the univoltine cohort showed higher intraspecific competition for *Artemia* nauplii (mild form of competition), but not resulting in cannibalism (extreme form of competition) [78].

We did not find support for our prediction of a lower survivorship in the early hatching group. Similar rates of survivorship between both phenology groups could be explained by the absence of strong competition, including cannibalism between different size cohorts. Size-dependent cannibalism caused by, for example, differences in hatching dates within rearing group, was reported in previous studies [23,55,79,80]. Our results support the theoretical prediction indicating that the outcomes of interactions between individuals from different cohorts with different trait values depends less on the hatching dates and more on other population characteristics, e.g., population density [81]. In our study both cohorts were present within each phenology group. Differences in development rates between overlapping cohorts might have reduced initial differences in larval sizes caused by different hatching dates between early and late hatched groups, which covered about 8% of the growth season [48]. Overlapping cohorts within each of the hatching phenology

group could weaken hatching phenology effects on life history traits, as it likely happened in the current study.

In ectotherms, an increased temperature may imply an increased immune challenge because of increased activity and growth of pathogens [43,82–84]. However, in our study no specific immune challenge was posed. In *I. elegans*, increasing temperatures up to 30 °C can increase the investment in immune function [85]. Such increase in PO activity was observed in early hatched females reared under warming. The increased PO activity at the higher temperatures might be caused by relocation of resources from other physiological traits such as energy reserves [42,86], but this needs further study for confirmation. However, this positive effect of warming on immune function might be paired with a response to thermal stress resulting in, for example, decreased survival in ectotherms [87,88]. Our results suggest that advanced hatching due to warming might be beneficial for the damselfly, given the increased energy investment into immune function is not traded off with investments in other physiological functions.

We found the highest PO activity in early hatched females grown under experimental warming. Such response may be beneficial for females because an elevated immune function can lead to increased longevity, the major determinant of female reproductive success [89,90]. On the other hand, because investment in immune function is costly, males are expected to invest more in traits which lead to increased mating success, e.g., increased activity in searching for a mate, and investment in secondary sexual traits such as ornamentation [89–91]. Hence, advanced hatching due to warming would promote increased immune reaction to pathogens in both sexes, and more so in females than males.

In conclusion, our results demonstrate that under experimental warming early hatched *I. elegans* showed an increased investment in immune function that, in companion with increased development and growth rate, may indicate an adaptive response to climate change in the high-latitude, season-limited populations. Our findings highlight the importance of variable voltinism associated with cohort splitting, even among individuals with the same hatching period, and how this might affect key fitness traits of ectotherms in a warming world.

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Miejscowość i data: Łódź 28.02.2023
Imię i nazwisko: Mateusz Raczyński
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

OŚWIADCZENIE

Oświadczam, że w pracy Sniegula, S., Raczyński, M., Golab, M. J. & Johansson, F. 2020, Effects of predator cues carry over from egg and larval stage to adult life-history traits in a damselfly. *Freshwater Science* **39** mój udział polegał na: zaprojektowaniu i przeprowadzeniu eksperymentu, pozyskaniu materiału, oraz przygotowaniu manuskryptu.

Procentowy udział **w** publikacji: 30%

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(czytelny podpis współautora)

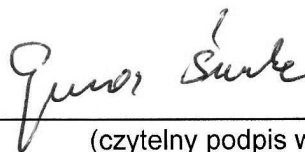Miejscowość i data: Kraków, 1-03-2023
Imię i nazwisko: Szymon Śniegula
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

OŚWIADCZENIE

Oświadczam, że w pracy Sniegula, S., Raczyński, M., Golab, M. J. & Johansson, F. 2020, Effects of predator cues carry over from egg and larval stage to adult life-history traits in a damselfly. *Freshwater Science* **39** mój udział polegał na: pozyskaniu funduszy, zaprojektowaniu i przeprowadzeniu eksperymentu, pozyskaniu materiałów, analizie danych, oraz przygotowaniu manuskryptu.

Procentowy udział w publikacji: 50%

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(czytelny podpis współautora)

Miejscowość i data: Kraków 15.02.2023
Imię i nazwisko: Maria J. Gołąb
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

OŚWIADCZENIE

Oświadczam, że w pracy Sniegula, S., Raczyński, M., Golab, M. J. & Johansson, F. 2020, Effects of predator cues carry over from egg and larval stage to adult life-history traits in a damselfly. *Freshwater Science* **39** mój udział polegał na: przeprowadzeniu eksperymentu oraz przygotowaniu manuskryptu.

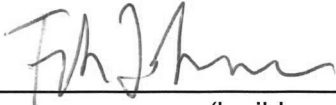Procentowy udział w publikacji: 10%

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(czytelny podpis współautora)

Location and date: Uppsala Febr. 15 2023
Name and surname: Frank Johansson
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DECLARATION

I declare, that in publication Sniegula, S., Raczyński, M., Golab, M. J. & Johansson, F. 2020, Effects of predator cues carry over from egg and larval stage to adult life-history traits in a damselfly. *Freshwater Science* **39,** my contribution consisted of: conceiving and designing the experiments and writing manuscript.

Percentage contribution: 10%

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(legible co-author signature)

Miejscowość i data: Łódź 28.02.2023
Imię i nazwisko: Mateusz Raczyński
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

OŚWIADCZENIE

Oświadczam, że w pracy Raczyński, M.; Stoks, R.; Johansson, F.; Sniegula, S. 2021 Size-mediated priority effects are trait-dependent and consistent across latitudes in a damselfly. Oikos, **130**, 1535–1547, mój udział polegał na: zaprojektowaniu metodologii, analizie formalnej, przeprowadzeniu eksperymentu, nadzorze nad danymi i analizie statystycznej, przedstawieniu graficznym, przygotowaniu pierwotnego manuskryptu oraz jego modyfikacji.

Procentowy udział w publikacji: 55%

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(czytelny podpis współautora)

Location and date: Leuven, 16 February 2023
Name and surname: Robby Stoks
Affiliation: Laboratory of Evolutionary Stress Ecology and Ecotoxicology, University of
Leuven

D E C L A R A T I O N

I declare, that in the publication "Raczyński, M.; Stoks, R.; Johansson, F.; Sniegula, S.
2021 Size-mediated priority effects are trait-dependent and consistent across latitudes in a
damselfly. Oikos, **130**, 1535–1547"**,** my contribution consisted of: formal analysis,
conceiving methodology, software support, writing original draft, review and editing of the
draft.

Percentage contribution: 10%

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(legible co-author signature)
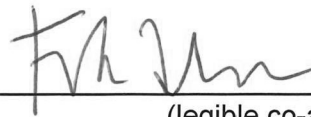
Location and date: Uppsala Febr. 15 2023
Name and surname: Frank Johansson
Affiliation: Department of Ecology and Genetics, Uppsala University

DECLARATION

I declare, that in publication Raczyński, M.; Stoks, R.; Johansson, F.; Sniegula, S. 2021 Size-mediated priority effects are trait-dependent and consistent across latitudes in a damselfly. Oikos, **130**, 1535–1547, my contribution consisted of: writing original draft, review and editing of the draft.

Percentage contribution: 5%

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(legible co-author signature)

Miejscowość i data: 1-03-2023, krakow
Imię i nazwisko: Szymon Śniegula
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

O Ś W I A D C Z E N I E

Oświadczam, że w pracy Raczyński, M.; Stoks, R.; Johansson, F.; Sniegula, S. 2021 Size-mediated priority effects are trait-dependent and consistent across latitudes in a damselfly. Oikos, **130**, 1535–1547, mój udział polegał na: pozyskaniu funduszy, zaprojektowaniu eksperymentu i metodologii, analizie formalnej i statystycznej, przeprowadzeniu eksperymentu, nadzorze nad projektem oraz eksperymentem, przedstawieniu graficznym, przygotowaniu pierwotnego manuskryptu oraz jego modyfikacji.

Procentowy udział w publikacji: 30%

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(czytelny podpis współautora)

Miejscowość i data: Łódź 28.02.2023
Imię i nazwisko: Mateusz Raczyński
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

OŚWIADCZENIE

Oświadczam, że w pracy Raczyński, M., Stoks, R. & Sniegula, S. 2022, Warming and predation risk only weakly shape size-mediated priority effects in a cannibalistic damselfly. *Scientific Reports* **12**, 17324 , mój udział polegał na: zaprojektowaniu metodologii, analizie formalnej, przeprowadzeniu eksperymentu, nadzorze nad danymi i analizie statystycznej, przedstawieniu graficznym, przygotowaniu pierwotnego manuskryptu oraz jego modyfikacji.

Udział procentowy w publikacji: 65%

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Location and date: Leuven, 16 February 2023
Name and surname: Robby Stoks
Affiliation: Laboratory of Evolutionary Stress Ecology and Ecotoxicology, University of Leuven

D E C L A R A T I O N

I declare, that in the publication "Raczyński, M., Stoks, R. & Sniegula, S. 2022, Warming and predation risk only weakly shape size-mediated priority effects in a cannibalistic damselfly. *Scientific Reports* **12**, 17324", my contribution consisted of: conceiving methodology, investigating, gathering resources, writing, review and editing of the original draft.

Percentage contribution: 10%

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Miejscowość i data: Kraków, 1-03-2023
Imię i nazwisko: Szymon Śniegula
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

OŚWIADCZENIE

Oświadczam, że w pracy Raczyński, M., Stoks, R. & Sniegula, S. 2022, Warming and predation risk only weakly shape size-mediated priority effects in a cannibalistic damselfly. *Scientific Reports* **12**, 17324, mój udział polegał na: pozyskaniu funduszy, zaprojektowaniu eksperymentu i metodologii, analizie formalnej i statystycznej, przeprowadzeniu eksperymentu, pozyskaniu materiałów nadzorze nad projektem oraz eksperymentem, przedstawieniu graficznym, przygotowaniu pierwotnego manuskryptu oraz jego modyfikacji.

Udział procentowy w publikacji: 25%

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Miejscowość i data: Kraków 28.02.2023
Imię i nazwisko: Mateusz Raczyński
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

O Ś W I A D C Z E N I E

Oświadczam, że w pracy Raczyński, M.; Stoks, R.; Johansson, F.; Bartoń, K.; Sniegula, S. Phenological Shifts in a Warming World Affect Physiology and Life History in a Damselfly. *Insects* **2022**, *13*, 622, mój udział polegał na: zaprojektowaniu metodologii, analizie formalnej i statystycznej, przeprowadzeniu eksperymentu, nadzorze nad danymi, pozyskaniem materiałów, przedstawieniu graficznym, przygotowaniem pierwotnego manuskryptu oraz jego modyfikacji.

Procentowy udział w publikacji: 55%

_____
(czytelny podpis współautora)

Location and date: Leuven, 16 February 2023
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Affiliation: Laboratory of Evolutionary Stress Ecology and Ecotoxicology, University of Leuven

D E C L A R A T I O N

I declare, that in the publication "Raczyński, M.; Stoks, R.; Johansson, F.; Bartoń, K.; Sniegula, S. Phenological Shifts in a Warming World Affect Physiology and Life History in a Damselfly. *Insects* **2022**, *13*, 622", my contribution consisted of: conceiving methodology, investigating, gathering resources, review and editing of the original draft.

Percentage contribution: 10%

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(legible co-author signature)

Location and date: Uppsala Febr. 15 2023
Name and surname: Frank Johansson
Affiliation: Department of Ecology and Genetics, Uppsala University

D E C L A R A T I O N

I declare, that in publication Raczyński, M.; Stoks, R.; Johansson, F.; Bartoń, K.; Sniegula, S. Phenological Shifts in a Warming World Affect Physiology and Life History in a Damselfly. *Insects* **2022**, *13*, 622, my contribution consisted of: conceiving methodology, investigating, gathering resources, review and editing of the original draft.

Percentage contribution: 5%

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(legible co-author signature)

Miejscowość i data: Kraków, 17.02.2023
Imię i nazwisko: Kamil Bartoń
Afiliacja: Instytut Ochrony Przyrody Polskiej Akademii Nauk w Krakowie

O Ś W I A D C Z E N I E

Oświadczam, że w pracy Raczyński, M.; Stoks, R.; Johansson, F.; Bartoń, K.; Sniegula, S. Phenological Shifts in a Warming World Affect Physiology and Life History in a Damselfly. *Insects* **2022**, *13*, 622, mój udział polegał na: analizie formalnej i statystycznej oraz modyfikacji pierwotnego manuskryptu.

Procentowy udział w publikacji: 5%

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Miejscowość i data: Kraków, 1-03-2023
Imię i nazwisko: Szymon Śniegula
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OŚWIADCZENIE

Oświadczam, że w pracy Raczyński, M.; Stoks, R.; Johansson, F.; Bartoń, K.; Sniegula, S. Phenological Shifts in a Warming World Affect Physiology and Life History in a Damselfly. *Insects* **2022**, *13*, 622, mój udział polegał na: pozyskaniu funduszy, zaprojektowaniu eksperymentu i metodologii, analizie formalnej i statystycznej, przeprowadzeniu eksperymentu, pozyskaniu materiałów nadzorze nad projektem oraz eksperymentem, przedstawieniu graficznym, przygotowaniu pierwotnego manuskryptu oraz jego modyfikacji.

Procentowy udział w publikacji: 25%

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