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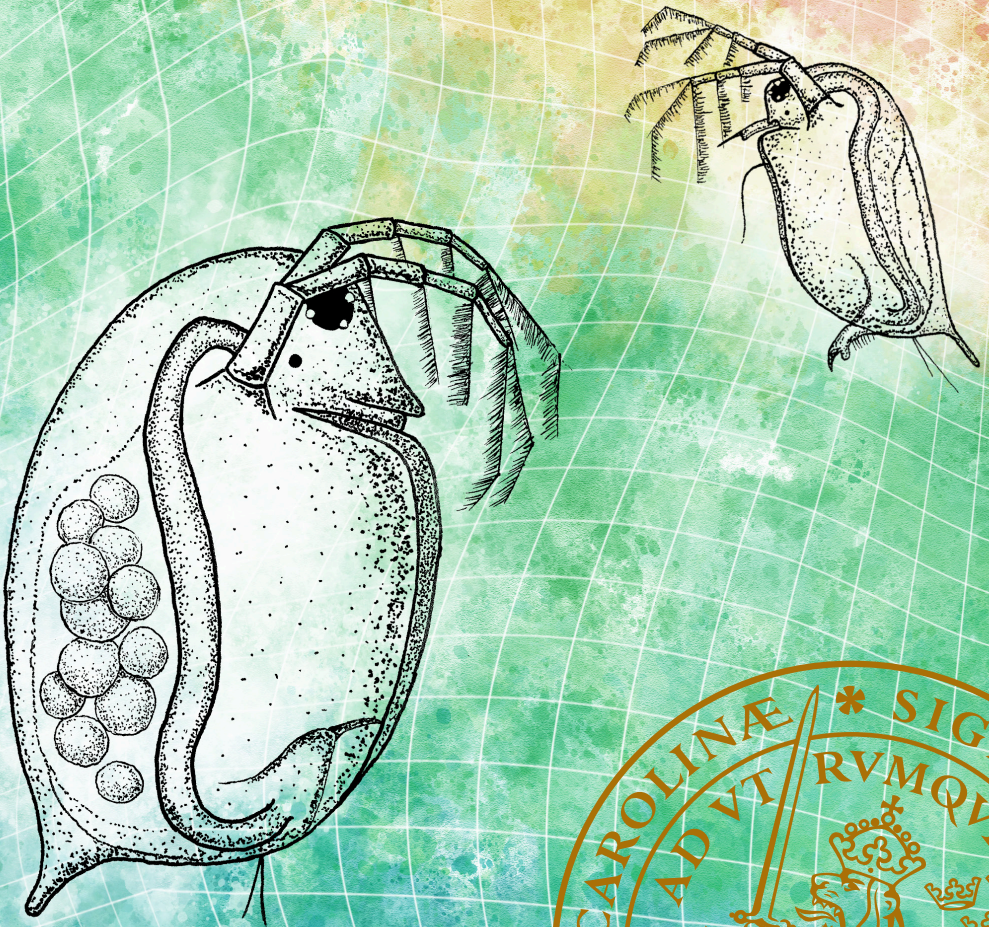
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Phenotypic responses of zooplankton to variable conditions

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Phenotypic responses of zooplankton to variable conditions

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Marcus Lee



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DOCTORAL DISSERTATION

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Associate Professor Matthew Walsh
Department of Biology
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<p>Organisms are continually challenged by multiple threats in the environment, and such threats are seldom constant in either time or space. Therefore, organisms must maintain physiological, behavioural, morphological and life-history adaptations across environments to prevent reductions in fitness. Freshwater ecosystems are particularly variable environments, and so organisms inhabiting lakes and ponds exhibit a range of different adaptations in order to survive and propagate. Despite this, the energetic constraints and potentially divergent responses required towards multiple threats creates the necessity to make trade-offs in their phenotypic repertoire. The outcomes of such phenotypic compromises are often difficult to predict and requires both a mechanistic understanding of the threats and responses involved, as well as insights into the resulting fitness consequences. Although this is relatively well understood with regards to some threats like predation for example, other biotic and abiotic stressors are less well studied. In this thesis I identify phenotypic compromises in behavioural, morphological and life-history traits of zooplankton with regards to variable environments.</p> <p>Behaviour is often considered one of the most labile traits and consequently it may provide a quick and inexpensive response to infrequent or rapidly changing contexts. Using 3D tracking, I focused on swimming behaviour of <i>Daphnia magna</i> in response to social context and found that females avoid males in a way that resembles a predation event. In a separate experiment I investigated how the presence of a high-density food patch altered the response to the abiotic threat ultraviolet radiation (UVR). Here I found that despite the potential fitness consequences of UVR the foraging opportunity reduced the avoidance response that has been classically described in <i>Daphnia</i>.</p> <p>UVR is a substantial threat in aquatic systems with documented physiological, behavioural, morphological and life-history responses. It is also a particularly variable stressor as it is absent during night and varies in intensity over the seasons. Despite its variable nature, studies have typically only addressed the presence or absence and not fluctuating UVR stress. I addressed this gap in the knowledge by tracking the survival, reproduction and behavioural response to both fluctuating and stable exposure of UVR. Simply by varying the scheduling but not the quantity of UVR stress, I identified fitness costs that appeared to be linked to the cost of the behavioural avoidance.</p> <p>In high-latitude environments, where phenotypic plasticity is promoted due to seasonal variability, copepods increase pigmentation in response to increased UVR but only in the absence of fish. I tested whether this response is ubiquitous at lower latitudes that experience less seasonality and have evolved with different predation regimes. Copepods from fishless environments had higher pigmentation than those with visually hunting predators. I also found that plasticity towards UVR removal was minor, but plasticity towards predation was mostly idiosyncratic. This suggests that plasticity does exist for the threats that are most variable and constitutive responses may have evolved towards ever present danger</p>			
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Phenotypic responses of zooplankton to variable conditions

Marcus Lee



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MADE IN SWEDEN 

To all my family

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- I. Lee, M., Solano Udina, C. & Hansson, L.-A. 2021 Fear of sex: sexual conflict exposed as avoidance in a parthenogenetic invertebrate. *Behavioural Ecology and Sociobiology* 75, 115. <https://doi.org/10.1007/s00265-021-03054-9>
- II. Lee, M., Hansson, L.-A. *Daphnia magna* trade-off safety from UV radiation for food. Manuscript (*Ecology and Evolution, in press*)
- III. Ståbile F., Brönmark C., Hansson L.-A., Lee M. 2021 Fitness cost from fluctuating ultraviolet radiation in *Daphnia magna*. *Biology Letters* 17: 20210261. <https://doi.org/10.1098/rsbl.2021.0261>
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Author contributions

- I. All authors conceived the project and contributed to writing. CSU and ML collected data. ML performed the data analysis and wrote the first version of the manuscript.
- II. ML and L-AH conceptualized and planned the study. ML carried out the study, performed the statistical analysis and drafted the first version of the manuscript. L-AH and ML both contributed to revisions.
- III. FS, CB, L-AH and ML conceived and designed the experiments. FS and ML conducted the experiments and analysed the results. FS wrote the first version of the manuscript with contributions from all co-authors. All authors edited, read and approved the manuscript.
- IV. ML, HZ, YS, AH and L-AH conceived and led the study. ML, RBL and PAN conducted the analyses. ML wrote the first version with substantial contributions from RBL and CB. All authors (GEU, JV, MŠ, VP, SDH, CaB, RG, EJ, NH, KH, KR) aided in fieldwork and provided valuable guidance both during the research process and the revision of the manuscript.

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Abstract

Organisms are continually challenged by multiple threats in the environment, and such threats are seldom constant in either time or space. Therefore, organisms must maintain physiological, behavioural, morphological and life-history adaptations across environments to prevent reductions in fitness. Freshwater ecosystems are particularly variable environments, and so organisms inhabiting lakes and ponds exhibit a range of different adaptations in order to survive and propagate. Despite this, the energetic constraints and potentially divergent responses required towards multiple threats creates the necessity to make trade-offs in their phenotypic repertoire. The outcomes of such phenotypic compromises are often difficult to predict and requires both a mechanistic understanding of the threats and responses involved, as well as insights into the resulting fitness consequences. Although this is relatively well understood with regards to some threats like predation for example, other biotic and abiotic stressors are less well studied. In this thesis I identify phenotypic compromises in behavioural, morphological and life-history traits of zooplankton with regards to variable environments.

Behaviour is often considered one of the most labile traits and consequently it may provide a quick and inexpensive response to infrequent or rapidly changing contexts. Using 3D tracking, I focused on swimming behaviour of *Daphnia magna* in response to social context and found that females avoid males in a way that resembles a predation event. In a separate experiment I investigated how the presence of a high-density food patch altered the response to the abiotic threat ultraviolet radiation (UVR). Here I found that despite the potential fitness consequences of UVR the foraging opportunity reduced the avoidance response that has been classically described in *Daphnia*.

UVR is a substantial threat in aquatic systems with documented physiological, behavioural, morphological and life-history responses. It is also a particularly variable stressor as it is absent during night and varies in intensity over the seasons. Despite its variable nature, studies have typically only addressed the presence or absence and not fluctuating UVR stress. I addressed this gap in the knowledge by tracking the survival, reproduction and behavioural response to both fluctuating and stable exposure of UVR. Simply by varying the scheduling but not the quantity of UVR stress, I identified fitness costs that appeared to be linked to the cost of the behavioural avoidance.

In high-latitude environments, where phenotypic plasticity is promoted due to seasonal variability, copepods increase pigmentation in response to increased UVR but only in the absence of fish. I tested whether this response is ubiquitous at lower latitudes that experience less seasonality and have evolved with different predation regimes. Copepods from fishless environments had higher pigmentation than those with visually hunting predators. I also found that plasticity towards UVR removal was minor, but plasticity towards predation was mostly idiosyncratic. This suggests that plasticity does exist for the threats that are most variable and constitutive responses may have evolved towards ever present danger.

Sammanfattning på svenska

Alla organismer, från den anspråkslösa daggmasken till den majestätiska eken, upplever stress. I biologiska termer betyder stress en minskad förmåga att överleva och fortplanta sig. Stress kan orsakas av en mängd olika faktorer som predation, svält och ultraviolett strålning för att bara nämna några. Djur måste hitta sätt att hantera dessa stressfaktorer, bland annat igenom att helt enkelt undvika faran, anpassa antalet avkommor de producerar eller utveckla en ekologisk motsvarighet till solskydd. Svårigheten med att anta en viss strategi beror på hur förutsägbar eller oförutsägbar var och en av de olika påfrestningarna är.

Sötvattenhabitat är ofta särskilt varierande och detta utgör en utmaning för alla organismer som lever i sjöar och dammar. Under vintern kan de frysa till is, på våren kan de vara så klart vatten att de leder till en hög nivå av ultraviolett strålning från solen tränger djupt ned i vattnet, under sommaren kan det produceras ett överflöd av giftiga alger, eller så kan vattnet torka ut helt! Därför har alla djur som lever i dessa miljöer unika anpassningar för att överleva, men de måste också bedöma de rådande förhållandena och antingen investera i en strategi som är fördelaktig vid just den tidpunkten, eller utveckla en strategi som kommer att vara fördelaktig i en framtida miljö.

Beteendeförändringar kan ske nästan omedelbart och kan därför vara en väldigt användbar strategi för att undvika farliga eller oförutsägbara omständigheter. Jag undersökte därför beteenderesponser under olika förhållanden hos ett ryggradslöst djur i mm-storlek som är både vanligt och viktigt i sjöar och dammar. I sin miljö kan vattenloppan (*Daphnia magna*) existera som enkönad. Med andra ord, under en större del av året finns endast honor som förökar sig genom "jungfrufödelse", men senare under säsongen produceras även hanar. Eftersom hanar inte är nödvändiga för att fortplanta sig kan detta skapa en intressekonflikt mellan könen. Jag visar i denna avhandling att honor, när de lever under gynnsamma förhållanden, reagerar på hanar på ett liknande sätt som om de utsattes för hot från ett rovdjur. Jag visar också att *Daphnia* är mindre benägna att söka skydd från ultraviolett strålning när det finns mycket mat i miljön, men att de starkt undviker strålningen under förhållanden med låg mat. Beteendereaktioner är dock inte utan kostnader och jag visar också i denna avhandling att det är växlingarna, snarare än mängden, av en stress som gör att vattenlopporna inte

producerar lika många avkommor. Denna minskning verkar vara kopplad till *antalet* flyktrörelser djuren tvingas göra som svar på hotet.

Andra sötvattensdjur använder pigment som svar på ultraviolett strålning, något som fungerar som ett solskydd. Men att vara färgglad gör dig också till en lätt måltavla för rovdjur. Därför kan de justera nivån av pigment för att anpassa sig till den miljö de lever i. Jag har visat att denna förmåga att reglera pigment inte bara är begränsad till miljöer som tidigare studerats, utan också finns kvar i miljöer där exponeringen av stressen alltid är varierande. I min avhandling visar jag ett litet urval av bredden av potentiella responser hos små sötvattensdjur, och hur viktigt det är att ta hänsyn till växlingar i stress för att förstå reaktionerna hos denna viktiga, men ofta bortglömda djurgrupp.

Introduction

All organisms are subject to multiple selective pressures, and the extent to which they can contend with such threats determines the success of the species. Not only are there multiple threats, but all threats and their risk vary in intensity over both space and time creating a highly dynamic environment to live in. Therefore, understanding how organisms respond to fluctuating environments is a fundamental topic in both ecology and evolutionary biology (Bell 2010, Franch-Gras et al. 2017, Bernhardt et al. 2020).

Threats and Risk

It is important to first understand the terms threat and risk. Threat refers to a factor in the environment that can cause stress, which is typically considered in the evolutionary terms of a reduction in survival and reproduction (Schulte 2014). Threats can be both biotic and abiotic in nature, for example, threats could be predation (Zanette et al. 2011), starvation (Kawecki 2010), competition (Pettay et al. 2016), salinity (Hall and Burns 2001), temperature (Mironidis and Savopoulou-Soultani 2010), ultraviolet radiation (UVR) (Williamson et al. 1994) or pathogens (Hart 1990). This is by no means an exhaustive list and threats will invariably depend on the focal organism. Risk on the other hand, is simply the likelihood that a threat will actually occur and thereby impose a cost on the organism (Campbell and Bartell 1998). It is important to distinguish between threats and risks because in environments where there are multiple threats, the likelihood of risk for each threat will determine the response of the individual. However, the relationship between the threat, the organism's perceived risk, and the organism's response are often imperfect leading to various mismatches (Gaynor et al. 2019)(Figure 1). As a result, selection has favoured the evolution of behavioural, morphological and life-history adaptations of organisms in response to fluctuations of threats and their perceived risks (LaManna and Martin 2016).

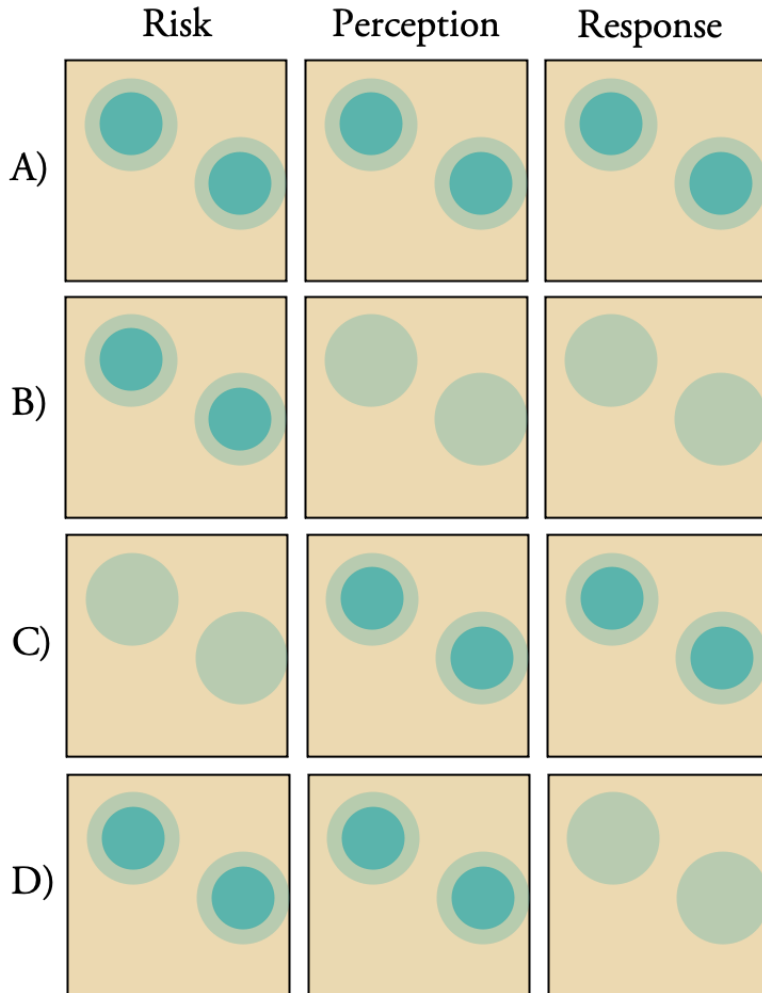


Figure 1. The spatial distribution of threats creates a heterogenous landscape of risk which may or may not match the individuals' perception of said risk. This leads to various scenarios where the actual risk, the perception of risk and the response differ. A) The ideal scenario for an individual is the perfect alignment between risk perception and response. B) shows an underestimation of the risk therefore responds weakly. C) shows an overestimation and consequently responds strongly. D) A common scenario may be the correct assessment of the risks yet the response is muted due to phenotypic constraints or high cost of responding. This is but a selection of many potential mismatches between risk, perception and response. Adapted from Gaynor et al. (2019).

Risk calculations are well understood in terms of foraging behaviours. In foraging ecology, food patches should be abandoned when the benefits no longer compensate for the costs of foraging, such as metabolism, predation risk and missed opportunities (Brown 1992). As an organism moves through an environment, from patch to patch, they are continually supplied with information of the environment. A recent concept has coined this perpetually updated spatial variation in perceived risk as the Landscape

of Fear (LOF) (Gaynor et al. 2019). The LOF has been proposed as an ecological framework that helps focus research of non-consumptive effects and has the potential to extend beyond the typical terrestrial mammalian study systems to be a useful tool in other taxa and aquatic systems (Wirsing and Ripple 2011, Laundré et al. 2014). Typically, this concept has been firmly couched in predator-prey interactions, as it has important implications for understanding the mechanistic controls of population ecology, of which predators are an immense regulatory pressure. It suggests that, as perceived safety diminishes and perceived risk increases, bottom-up impacts i.e., resource limitation, reduce and top-down control i.e., predation, mechanisms become more influential (Laundré et al. 2014). However, as detailed above, predators are not the only threat in the environment and by considering any potentially fitness reducing stimuli, the LOF becomes a more unifying concept than in its current use.

As a response to the perceived threats in the environment, mobile organisms will often attempt to reduce the risk via avoidance. This links to a well-established predator sequence which begins with the probability of being detected, then the probability of an attack, and finally the probability of the attack leading to death for that individual (Brönmark and Hansson 2018). The prey organism in this scenario can employ many defences to alter the probabilities of any step in that sequence, and a particularly effective solution is to completely avoid the threat if perceived. However, the use of various biologging equipment have highlighted how prey and their predators do not always exhibit strong spatiotemporal avoidance (Cusack et al. 2020), yet the root cause of this lack of avoidance is unclear. As aided by the framework of the LOF, the overlap may be due to a mismatch of risk and perception, perception and response or it could be that migration from a high-risk area is associated with substantial costs that prohibit such a response (Gaynor et al. 2019). Alternatively, as the predation cycle has multiple steps at which inducible defences can be beneficial, multiple forms of defences can be selected for. For example, if an organism can predict where the greatest risk is, through the sensing of reliable cues, avoidance will likely benefit that organism greatly. However, if the cues are variable, defences that decrease a predators' success post-contact may be more beneficial (Riessen and Trevett-Smith 2009). Therefore, phenotypic plasticity is an alternative and complimentary adaptation that can mitigate risks in the environment.

Plasticity

Some disambiguation is required as phenotypic plasticity has historically been used rather liberally to describe multiple phenomena, such as maternal effects, developmental plasticity, and inducible defences among others (Forsman 2015). Phenotypic plasticity, at its most basal, is the ability of one genotype to express multiple

phenotypes (Figure 2). This property has been identified for over a century (Woltereck 1909), however the terms genotype and phenotype were originally coined a few years later (Johannsen 1911). Since its identification, plasticity has been documented in almost all taxa, and therefore such ubiquity suggests it is a particularly important aspect of evolution. A recent and particularly succinct history of plasticity was detailed by Sommer (2020), which draws attention to the historical resistance towards this concept. Ignoring plasticity can lead to drastic oversights when considering local adaptation and contemporary trait evolution, as adaptive trait changes have been demonstrated to have strong alterations in community structure (Peacor et al. 2012, Burak et al. 2018).

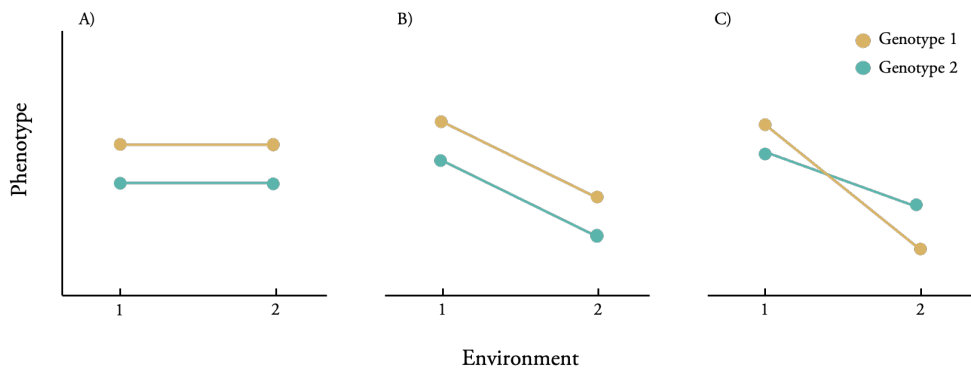


Figure 2. Phenotypic plasticity is typically represented by a reaction norm, whereby the phenotypic expression of a genotype is displayed across environments. A) represents phenotypic variation originating from genetic variation i.e., no plasticity, B) shows genetic variation and plasticity, and C) highlights genetic variation in plasticity.

The evolution of adaptive plasticity requires a few assumptions; (1) the environmental threat should be an important mortality source and the threat is selective in terms of the alternative phenotype, (2) there are reliable cues of the threat, (3) there is either spatial, temporal or both forms of variation in the threat and (4) the alternative phenotype provides a benefit in the presence of the threat yet demands a cost in its absence, else a constitutive defence is likely to evolve (Tollrian and Harvell 1999).

The major benefit of plasticity is rooted in the ability for organisms to maintain fitness across multiple environments. When encountering a stressful environment, a mobile organism may be able to move to a more favourable environment, as discussed above. However, not all organisms can move, or move fast enough to track the optimum environment and so are subject to selection. Eventually selection will likely generate a phenotype that is adapted to the current environment, but should that environment change then the process begins again (Bell 2010). Alternatively, phenotypic plasticity allows an organism the ‘best of both worlds’. In a particular environment they can express the phenotype beneficial there, and when in the alternative environment they

can express a better suited phenotype. It is important to see plasticity and genetic evolution as complementary processes as plasticity can also rapidly evolve (West-Eberhard 2003); a view that is well supported by empirical evidence demonstrating genotype specific effects of both within and across generation plasticity, highlighting the potential for local adaptation (Walsh et al. 2016, Reger et al. 2018, Sha et al. 2020a).

In order to generate an alternative phenotype, the genotype must receive information regarding the environmental conditions or threats i.e. ‘cues’. Models have shown how the periods in which organisms are exposed to cues can have dramatic effects on the phenotype exhibited (Panchanathan and Frankenhuis 2016). Developmental plasticity, whereby cues are introduced during ontogeny and shape the resulting phenotype, can be irreversible but also may simply be too slow to benefit from reversals. For example, freshwater snails (*Helisoma trivolvis*) form morphological defences to a predatory water bug (*Belostoma flumineum*) and the timing of exposure to predation risk determined if the snail was able to reverse the defence in the absence of the threat (Hoverman and Relyea 2007). This demonstrates that the variability of the environment can impact the utility of developmental plasticity, even producing maladaptive phenotypes i.e., mismatches between the phenotype and the current environment. Reversible plasticity such as behavioural modifications may counteract such negative consequences (Beaman et al. 2016). This is particularly relevant when exposed to threats that are temporally or spatially variable within a generation or over the ontogeny of an individual (Riessen and Trevett-Smith 2009).

If cues are reliable and indicate the predictability or autocorrelation of the environment between generations, theory suggests the evolution of anticipatory maternal effects or transgenerational plasticity (Agrawal et al. 1999, McNamara et al. 2016, Lind et al. 2020). Despite certain experimental examples of maternal effects, such as maternal age having a significant impact on offspring life-history (Plaistow et al. 2015), meta-analyses have revealed only weak support of adaptive/anticipatory parental effects (Uller et al. 2013, Radersma et al. 2018). An interesting possibility is that individual variation may dampen such effects (Gillis and Walsh 2019). Individual variation in plasticity may occur due to various intrinsic variables such as size, energetic state or age which alters the cost-benefit analysis of plastic responses (Houslay et al. 2020), or it could represent a third complimentary strategy: bet-hedging.

Bet-Hedging

Even in genetically identical individuals, raised in essentially identical environments, there is often variability in their phenotype at the physiological, morphological and behavioural levels (Scheiner 1993, Buchanan et al. 2015, Vogt 2015, Bruijning et al.

2020). In *Escherichia coli* bacteria, it has been demonstrated that, despite being isogenic, behavioural diversity is predominantly determined by individuality and to a lesser extent by plasticity (Pleška et al. 2021). Individual variation stems from developmental stochasticity, and this notion is over a century old (Warren 1902). Stochastic, by definition, refers to variables that cannot be predicted a priori (Honegger and de Bivort 2018), and for an organism unpredictability presents a challenge as to how best to adapt.

One option is to engage in bet-hedging. This term is often expressed as a trade-off between the mean and variance of a strategy (Starrfelt and Kokko 2012). The two most commonly discussed forms of bet-hedging are diversified and conservative bet-hedging. Diversified bet-hedging (DBH) is when a single genotype produces multiple offspring phenotypes, which in an unpredictable environment may allow some to survive (Childs et al. 2010). In contrast, conservative bet-hedging (CBH) is the reduction in variance of fitness among offspring through the selection of a less risky but sub optimal phenotype. DBH is readily invoked as an explanation for increased phenotypic variation (Figure 3), however to truly be a bet-hedging strategy both DBH and CBH must also be accompanied by a decrease in both mean fitness and variance in genotype-level fitness (Hopper 1999, Haaland et al. 2019). In other words, a decrease in current reproductive output with increased likelihood of an increase in long-term fitness.

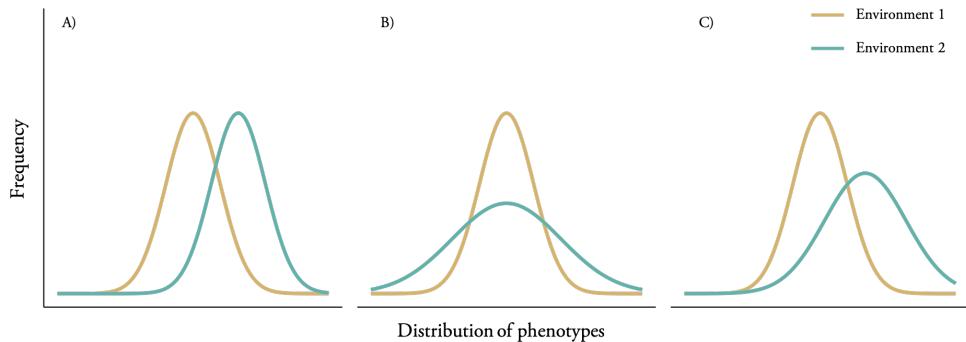


Figure 3. Plastic responses are typically considered in terms of the mean change in the phenotypic distribution of a population distribution (A), however an alternative response could be to increase the variance in phenotypic distributions, in other words spread the risk over many phenotypes (B). This strategy is often attributed to bet hedging. Mean changes and variance changes (C) are not mutually exclusive and regularly occur in tandem.

Plant and micro-organisms have provided strong experimental examples consistent with the bet-hedging hypothesis, however fewer examples are found in animals (Honegger and de Bivort 2018). Despite the paucity of examples in the animal kingdom, technological advancements increase the likelihood of studying such strategies in nature. For example, biologging has proven useful for identifying

behavioural individual variation, as not only is the average behaviour important, but also the level of plasticity and the predictability of behaviours are crucial to understanding the resilience of a population (Hertel et al. 2021). However, there are particular methodological constraints for studying individual variation. Ideally, to study the causes and consequences of individual variability an organism should reproduce clonally, live in simple and easily controlled environments and have a multiple easily measured traits (Vogt et al. 2008).

Zooplankton as a model group

Zooplankton is a general term referring to animals that drift in marine and freshwaters (Brönmark and Hansson 2018). They vary immensely in morphological features and span many taxonomic groups, with some only presenting as zooplankton before maturing (Kiørboe 2011, Everett et al. 2017). This group is a keystone in the trophic web, most obviously being the intermediate link between phytoplankton and higher trophic levels such as fish (Libralato et al. 2006, Richardson 2008), however they also provide key links to the microbial loop and therefore have a significant role in ecosystem functioning (Porter 1996). Due to their importance in aquatic systems, zooplankton have long been recognised as useful model systems for a variety of subjects such as ecotoxicology (Altshuler et al. 2011), foraging ecology (Dodson 1990, Dawidowicz and Loose 1992a, b, Burks et al. 2002), trophic interactions (Romare and Hansson 2003, Park and Post 2018), phenotypic plasticity (Woltereck 1909, Lampert 1993, Agrawal et al. 1999, Tollrian and Harvell 1999) and as genomic model organisms (Miner et al. 2012, Orsini et al. 2016) to name only a subset.

In freshwater ecosystems, the term zooplankton more commonly refers to one of the three major taxonomic groups that are nearly globally prevalent: Crustaceans, Rotifers and Protozoans (Thorpe and Covich 1991). Crustacean zooplankton, which are the focus of my research, are broadly divided further into the two predominant taxonomic orders: Cladocera and Copepoda. Both groups inhabit similar environments often being present simultaneously however, despite being exposed to the same threats, evolutionary history has equipped both groups with a separate suite of traits that create unique trade-offs between the phenotype and the perceived risk. Broadly speaking, *Daphnia spp.*, or 'water fleas' (perhaps the most well-known of the Cladocera) are larger bodied, voracious filter-feeders and more susceptible to predation than their copepod counterparts, which in turn are more selective feeders and being smaller, they are less apparent to visually hunting predators (Sommer and Sommer 2006, Brönmark and Hansson 2018). Such differences will inevitably directly affect their perception of risk and as such their spatial distribution within lakes.

Historically, zooplankton behaviour has relied on sampling methods that estimate differences in population density at separate time points to infer migration ranges. This however can be misleading to the behaviour of individuals within that population (Lampert 1989). Population dynamics are inherently governed by the behaviour of their constituents, and this highlights the importance of studying not only the population response, but the individual behavioural variation to threats (Ohman 1988). Similarly, the phenotypic plasticity of individuals has been demonstrated to scale up and effect the community composition (Peacor et al. 2012). Therefore, it is important we understand individual responses to various threats to develop a more comprehensive appreciation of life.

Aims of Thesis

The aim of my thesis was to investigate potential adaptive responses of zooplankton to various environmental stressors at the individual level, which may have important consequences for the spatial and temporal distributions of the population and subsequently the community. I have investigated how certain biotic and abiotic environmental components influence the behaviour, life history and morphology of freshwater crustacean zooplankton. Specifically, I aimed to address the following questions:

- (1) How does the social context influence space use in *Daphnia magna*? Do the potential costs of sex influence swimming behaviours, and if so, are the responses analogous to other threat avoidance strategies? (**Paper I**)

- (2) How does the opportunity of a high-density foraging patch influence the threat assessment of the abiotic stressor UVR? (**Paper II**)

- (3) How do *Daphnia* cope with natural fluctuations in UVR stress? Can behavioural plasticity compensate for negative fitness consequences of UVR stress? (**Paper III**)

- (4) Is phenotypic plasticity in pigmentation, that is highly prevalent at high latitudes, maintained at lower latitudes? Can copepods adjust their pigmentation to match the prevailing risk? (**Paper IV**)

Behaviour and biotic interactions

It has long been acknowledged that interspecific variation has important consequences for eco-evolutionary dynamics (Brooks and Dodson 1965, Post and Palkovacs 2009) which has dramatic effects on the surrounding environment. For example, the effects of trophic cascades in Yellowstone national park after the reintroduction of wolves to the ecosystem led to low abundances of elk and bison, which released many successional plant species from grazing pressure thereby altering the landscape (Beschta and Ripple 2016). In aquatic habitats the same principles have been highlighted through the presence of zooplankton herbivores increasing the amount of toxins produced by phytoplankton, which has the potential to structure marine ecosystems (Selander et al. 2019). More recently research has highlighted how intraspecific variation can also have profound consequences on the ecosystem through indirect effects (Des Roches et al. 2018). Behaviour is perhaps one of the most labile traits and consequently it is of particular importance to biologists attempting to explain the variation between individuals (Stamps et al. 2013).

Daphnia (and copepod) behaviour has been a topic of study for decades due to the notable daily migration that many species engage in, known as diel vertical migration (DVM). DVM is primarily considered as a foraging strategy whereby zooplankton avoid the surface waters during the day, minimising light-dependant mortality, and swim up at night to forage in the food-rich surface waters (Lampert 1989, Brierley 2014). As discussed briefly above, studies typically identify DVM by the differences in population density at separate time points, thereby inferring migration ranges. This can be misleading for the behaviour of individuals within that population (Lampert 1989). For example, it has been demonstrated that *Daphnia* are able to assess the prevailing threat and vertically stratify along a size structured gradient, indicating the capability to assess one's own current phenotype and adjust space-use within a population (Hansson and Hylander 2009b). Populations not only contain small and large specimens but also different sexes which may generate intersexual conflict and alter how the population is structured.

Intersexual conflict and swimming behaviours

For the majority of the growing season, *Daphnia* reproduce asexually (Figure 4), however when conditions deteriorate, typically due to high population density (Haltiner et al. 2020), they can switch to sexual reproduction. This switch between asexual to sexual reproduction in *Daphnia* (and other facultatively sexual organisms) is a key fitness factor in populations (Berg et al. 2001) as it allows the production of resting stages (ephippia). These sexually produced ephippia can remain viable for decades (Hairston et al. 1999, Gyllström and Hansson 2004). Sex is not a ubiquitously successful strategy however, there are multiple potential costs involved, including but not limited to the energetic costs of finding a mate, harassment (typically of females) and demographic costs of producing males (Gerber and Kokko 2016). Therefore, asexual reproduction is often considered the more efficient strategy as the potential for population growth is exponential and the cost of male production is removed (Gerber et al. 2018). There is clearly a delicate balance between fast population growth and population persistence, and the early onset of sexual reproduction could have critical consequences for a genotype as it can drastically reduce its representation in the population (Gerber et al. 2018). This likely creates divergent interests with males only gaining fitness through sex and females trying to maximise their representation in the environment via asexual reproduction.

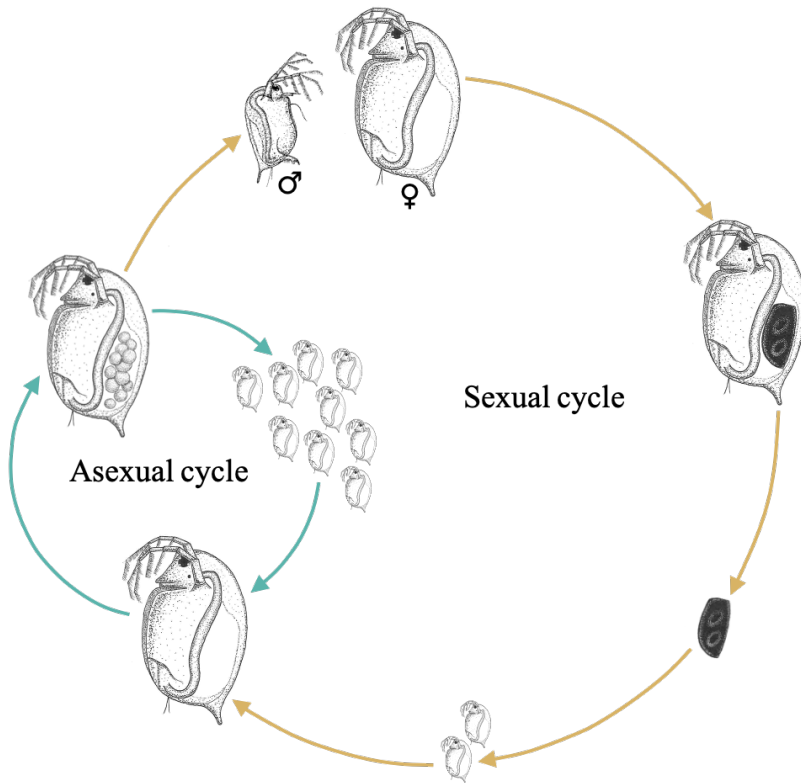


Figure 4. *Daphnia* life cycle. During the growing season *Daphnia* typically reproduce asexually, producing a high number (potentially in excess of 100) of genetically identical offspring. A female can also produce male *Daphnia*, which go on to mate with receptive females. The result of sex is an ephippium (diapausing/resting egg) developing instead of clonal daughters. This ephippium is released into the environment and can remain dormant for decades before hatching into a maximum of two offspring that grow into adults, capable of both forms of reproduction.

In **Paper I**, I investigated swimming behaviours of *Daphnia magna* males and females. Specifically, how swimming behaviours may change when exposed to either the same or opposite sex. As females can reproduce without males, but males' fitness is tied intimately to mating success, I hypothesised that females would avoid the potentially fitness-reducing males. Using an advanced 3D tracking platform and labelling individual zooplankton with fluorescent nanoparticles, I observed swimming behaviours of both males and females simultaneously. As expected, females avoided males by swimming deeper in the water column, and females increased their speed which taken together with the space-use indicates an escape response (Figure 5). Interestingly, this reaction is similar to the response to predators and ultraviolet radiation which suggests this behaviour is a general response. I also found, counter to the previous literature (Brewer 1998, La et al. 2014), a lack of sexual dimorphism in swimming behaviours with males and females swimming similarly when with the same

sex. This could be a species-specific difference with the older literature or this finding could highlight the importance of density and demography on swimming behaviours and space-use.

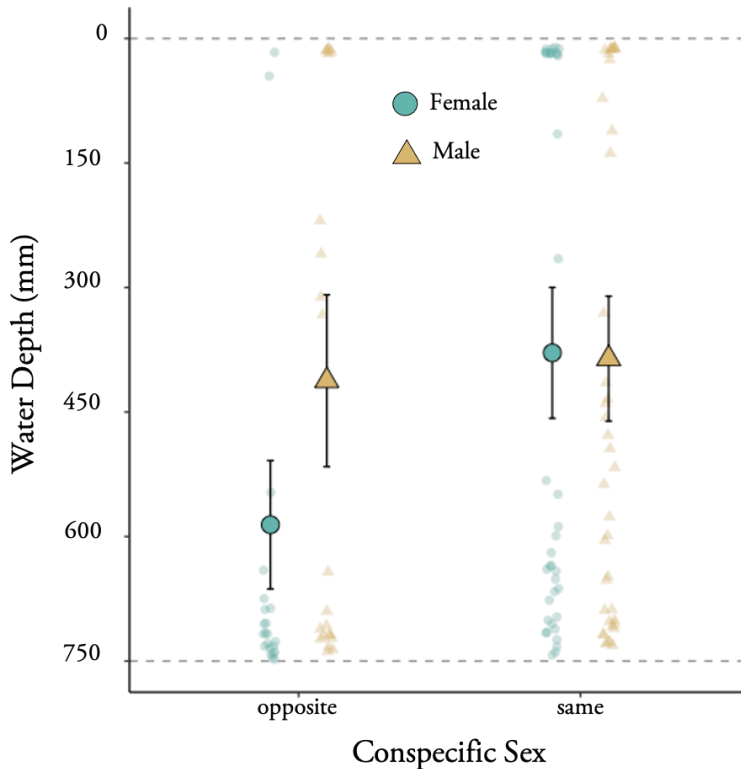


Figure 5. Effects of sex and conspecific sex on the average swimming depth in *Daphnia magna*. The large points represent the model estimates ± 2 SE with raw values (individuals) plotted as faded points. The dashed lines represent the boundaries of the water column

Biotic and abiotic interactions

Zooplankton use DVM as both a proactive and reactive response to minimise risk from multiple threats; predation risk being one of the major drivers in DVM in cladoceran and copepod taxa (Lampert 1989, Hays 2003, Sha et al. 2020b). Extensive work has been carried out in a vast array of zooplankton taxa with regards to the trade-off between food and predation (Johnsen and Jakobsen 1987, Dini and Carpenter 1992, Loose and Dawidowicz 1994, van Gool and Ringelberg 1998), however there are multiple threats and opportunities that influence the magnitude of this response. DVM

has been documented from environments where such predation was not a strong predictor (Muluk and Beklioglu 2005), indicating that other factors are important in the cost-benefit analysis when foraging. A comprehensive review published recently highlighted the number of external environmental variables that influence the amplitude of diel vertical migration (Bandara et al. 2021)

Unlike predation, abiotic threats tend to be more chronic than acute stressors. One such ubiquitous chronic threat, with both significant direct effects and complex indirect effects on ecosystems, is ultraviolet radiation (UVR). This is the section of solar radiation between the wavelengths 280 and 400 nm (Tucker and Williamson 2011). UVR has been shown to induce escape responses in many zooplankton (Rhode et al. 2001), however most studies have demonstrated this either in the field or carefully controlled lab experiments without the spatially explicit foraging opportunities as a counter. This may create an overestimation of the effects of UVR on spatial distribution as the fitness consequences of UVR are far more delayed than the effects of predation. In conjunction with the estimated risk of damage, photoprotective compounds such as mycosporine-like amino acids or carotenoids can be sequestered from algae (Moeller et al. 2005, Tartarotti and Sommaruga 2006) and so *Daphnia* may decide the opportunities outweigh the costs.

In **Paper II**, I address this gap in the available knowledge by testing how *Daphnia magna* respond when exposed to UVR in an environment with or without a foraging opportunity. Using the same system as with **Paper I**, I created a spatially explicit food patch at the surface of the water which creates a trade-off between opportunity for energetic gain and the highest risk or damage from UVR exposure above. In contrast to other studies that show a near ubiquitous negative phototactic response to UVR (Hansson et al. 2016, Fernández et al. 2018, Ekvall et al. 2020, Sha et al. 2020a), I demonstrate that the presence of a food patch reduces the average swimming depth when exposed to UVR. As the design of this experiment required removing the long acclimatisation phase that was present in other similar studies, we see that the initial response of many individuals to a novel environment is to swim down (Figure 6). However, looking at the average depth, many more individuals remain near the surface when presented with the foraging opportunities, and interestingly there is a small downward motion immediately followed by a return to the surface which indicates a reassessment of risk from the moment of entry to swimming out of the foraging patch.

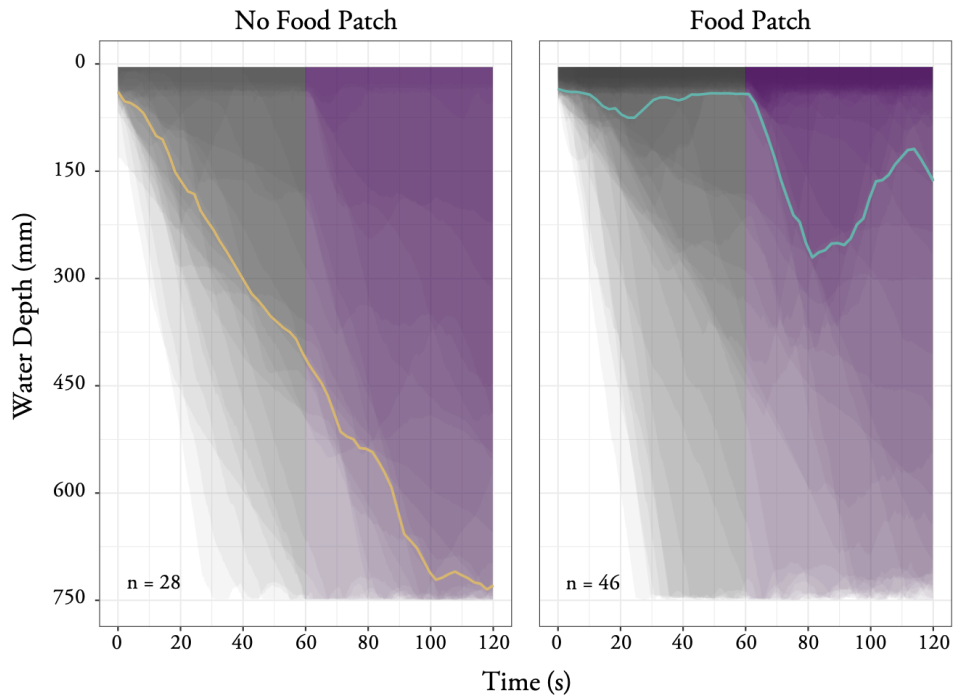


Figure 6. Individual swimming depths (areas plotted over one another) during experimental assays offering no feeding opportunity (left) and a food patch at the top (right). The median depths are indicated as coloured lines, where the blue line represents the median depth with an opportunity (food patch) and the yellow line shows the median without a surface feeding opportunity. The grey section (first 60 seconds) represents the acclimation phase and the purple (last 60 seconds) denotes when individuals were exposed to a UVR threat from above.

Aside from the environmental difference, there appears to be a great amount of individual variation in both treatments which mirrors both **Paper I** and other studies conducted with the same platform. This has previously been shown not to be simply a clonal artefact, but even isogenic females exhibit substantial variation, especially under exposure to a threat (Heuschele et al. 2017). There are multiple further non-exclusive influences that could explain this variation, but one highly likely factor involved in the decision to forage under risk is the individual's state. More specifically, foraging is a behaviour dependant not only upon the external risks, but also the individuals' energetic reserves. For instance, dogwhelks (*Nucella lapillus*) only increased their foraging activity when exposed to a predatory crab (*Carcinus maenus*) when starved (Matassa and Trussell 2014). Therefore, I anticipate the individuals studied could have very different internal states such as high lipid reserves or be actively reproducing. Further research can investigate whether this apparent individuality is a stochastic property or relates to an internal state variable that can account for a large portion of the variation observed.

Fluctuations of threats

In nature, the availability of opportunities, such as mates and food in the immediate vicinity, will vary both within and between generations. Similarly, threats such as predation, UVR and even mates (which can present a threat as documented in **Paper I**) vary on similar timescales to opportunities. This will create fluctuations in the perceived risk which can alter the optimal strategy. Typically, environmental fluctuations are often considered in terms of predictable and unpredictable elements (Franch-Gras et al. 2017). If cues are predictable but variable plasticity may be selected for. Alternatively, if the environment is variable but the signals are unpredictable bet-hedging may be a more effective strategy (Via and Lande 1985, Moran 1992, Simons 2011). Despite setting plasticity and bet-hedging as alternative strategies, it is important to consider them as potentially complementary as not only can a trait be plastic but the trait distributions can also be plastic and depend on environmental cues (Xue and Leibler 2018).

Consequences of constant vs fluctuating threats

UVR has been documented to have severe impacts on organisms, such as DNA damage, reduced growth, and reduced fecundity (Rautio and Tartarotti 2010). Therefore, there is strong pressure to avoid this environmental threat. In **Paper II** I demonstrated such a behavioural response, i.e. the avoidance to UVR. The simple action of responding to a threat is implicitly attached with costs, such as the energetic expenditure of movement and the missed opportunity for further foraging. These costs must constrict this behaviour of UVR avoidance to only when it is necessary, as to not outweigh the benefits of reduced exposure.

As with all environmental threats, UVR is both spatially and temporally variable. Much of the research on the UVR effects is concerned with the spatial aspect of this variation as UVR is attenuated by the water thereby providing a refuge deeper in the water column. Moreover, the transparency of water drastically alters the level of UVR and so studies have investigated these effects over space in different lakes and to a lesser extent, seasons (Hansson 2004, Williamson et al. 2011). Far less attention is paid to the effects

of UVR fluctuations that occur, not only regularly over a circadian cycle, but also vary strongly over short time scales, due to the variation in cloud cover for example.

I aimed in **Paper III** to investigate how short-term variability in UVR threats effected individuals over time. I exposed *Daphnia magna* to either no UVR, 6 hours of constant UVR or 6 hours of UVR fluctuating between on and off every 15 minutes for 45 days. I then tracked the survival and total reproductive output as a measure of fitness. The variable but potentially predictable scheduling of the threat allowed us to determine the effects of variability, but also test whether fitness could be increased through plasticity, i.e., learning (Stamps and Krishnan 2017). I found that survival remained similar among treatments but reproduction was reduced in the fluctuating environment only. This indicates that simply the temporal variation of the threat had fitness consequences, not the intensity. Interestingly, I found that over time there was no indication that *Daphnia* adjusted their behavioural response to UVR with increasing exposure. That is to say they did not exhibit behavioural plasticity. Despite the lack of plasticity over the experiment, it was obvious that those exposed to UVR constantly responded by remaining in the lower half of their aquarium for the duration of the UVR exposure (Figure 7). This indicates that the reduced fitness appears to stem from the trade-off between behavioural responses and reproductive output.

Despite not finding plasticity as expected, the behavioural result revealed multiple interesting possibilities. First, there were strong genotypic differences, which supports the potential for local adaptation. And second, those exposed to the fluctuating environment appeared to have more variation in their behavioural profiles than those in either constantly benign or stressful environments, at the same time as having reduced fitness. Superficially these characteristics resemble a diversified bet-hedging strategy, however I must make it clear that without following these individuals over generations I cannot confirm this. Instead, I wish to highlight the potential that the costs accrued in the fluctuating environment may indeed have been due to energy expenditure of responding to UVR or they may represent differential provisioning of offspring in order to maintain fitness across generations.

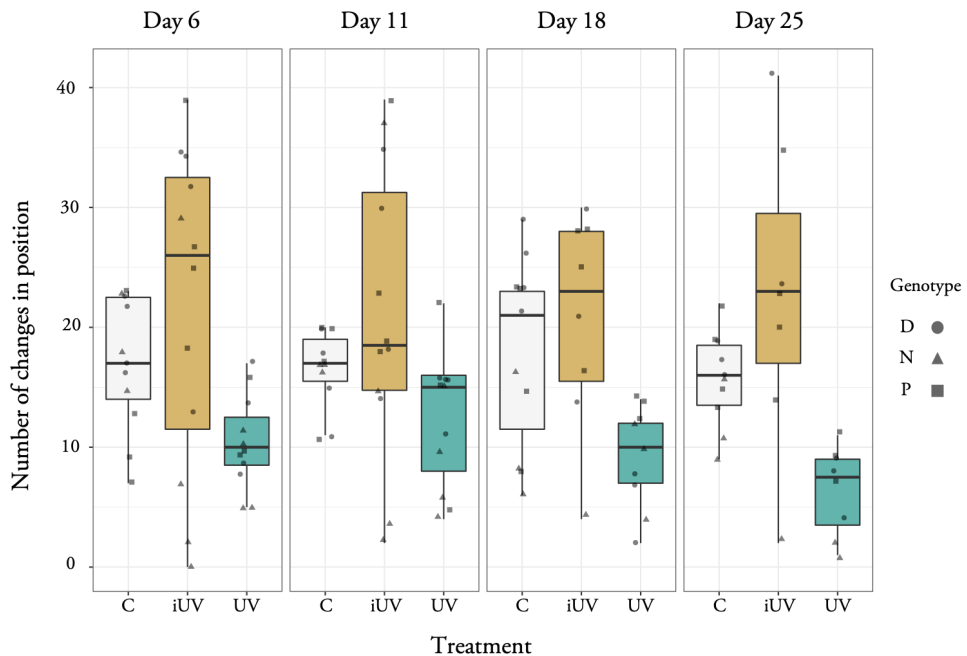


Figure 7. Number of changes in position between treatments for each behavioural recording day. C is the control group, i.e., no UVR but white light for 12 hours, iUV refers to 6 hours of UVR fluctuating over 12 hours and UV is the group exposed to 6 hours of stable UVR within 12 hours of white light. The grey symbols represent the data from each individual *Daphnia* and different-shaped dots indicate different genotypes. The boxplot shows the median for each group as a black horizontal line, the first and third quartile with the box, and the minimum and maximum with the whiskers.

In order to disentangle the potential strategies at play with **Paper III**, I have initiated a follow up experiment to examine the effects of fluctuating UVR over generations. Briefly, I have reared the same three genotypes as in **Paper III** under stable UVR for three generations. I have then taken the offspring from one brood, of one mother per genotype and split them into either stable UVR or fluctuating UVR environments to establish the experiment. From there, I am tracking the survival and reproductive output of each individual every day, across two generations. This allows me to assess the phenotypic changes in both mean and variance of multiple life history traits, such as reproductive maturity, neonate size and number of broods, among others, in both the fluctuating and stable but stressful environments. Preliminary results (not shown here) suggest that despite unstable environments having a fitness disadvantage in the parental generation, as observed in **Paper III**, the offspring do better than those from a stressful but stable environment. Early life mortality appears to be the cause of this, which has been documented previously (Huebner et al. 2006), but my results suggest that fluctuations in such a threat offer an opportunity to repair the damage caused by UVR. Also, this difference in fitness over generations partially fulfils the assumptions

of a bet hedging strategy, however as this experiment is ongoing at the time of writing, this may be subject to revision upon the collection of the full dataset.

Seasonal fluctuations and multiple threats

Threats obviously vary in space and time, but they also rarely occur in isolation. Certain threats can have convergent responses that minimise risk and maximise population growth (for example diel vertical migration as a response to both UVR avoidance and predation risk), however a potentially more common situation is where multiple threats require divergent responses. For example, predation and low-quality cyanobacterial food result in opposing life-history strategies (Whittington and Walsh 2015). Similarly, pigmentation is a common response to minimize damage from UVR however this increases the risk of visually hunting predators detecting them (Hansson 2000, Hylander et al. 2009)(Figure 8).

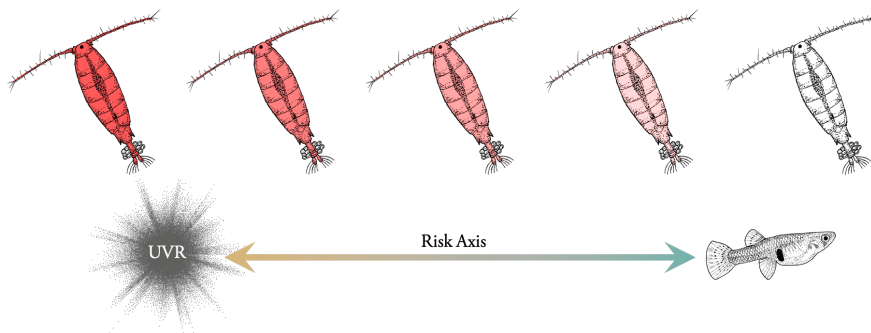


Figure 8. An illustration depicting the trade-off in pigmentation. On the left well-pigmented individuals are protected from UVR and vulnerable to predation, whereas those on the right are less visible to visually hunting vertebrate predators upon yet vulnerable to UVR damage. Adapted from Hansson and Hylander (2009a).

Specifically, when I discuss pigmentation, I am referring to carotenoids. Carotenoids are compounds that are synthesised by various taxa such as bacteria and algae, however zooplankton must sequester these pigments from their diets (Hansson and Hylander 2009a). Due to the antioxidant properties of carotenoids, they provide zooplankton protection against damaging radiation (Moeller et al. 2005) and in low-UVR systems they have been shown to be related with lipid storage (Schneider et al. 2016). There are clear taxonomic biases in relation to pigmentation as copepods often have up to ten times the level of carotenoids as their cladoceran counterparts which highlights the distinct adaptations different organisms have to address similar threats. Notably,

carotenoid pigmentation is a phenotypically plastic trait in crustacean zooplankton (Hansson 2000, 2004, Brüsin et al. 2016). This research has typically been conducted at high latitudes, where environmental variation is substantial and potentially selects for a more plastic strategy. Less is known about zooplankton and their adaptations at lower latitudes.

Therefore, in **Paper IV** I investigated the pigmentation level of copepods inhabiting various low-latitude 'blue holes' on The Bahamas. These blue holes are vertical caves that have filled with anoxic marine groundwater and have a freshwater lens at the surface (Björnerås et al. 2020). These environments are exposed to UVR year-round and vary in trophic complexity with some having no vertebrate predators, other having zooplanktivorous fish, and there are even blue holes with zooplanktivorous and piscivorous fish. I determined the pigmentation level of copepods from each of the predation regimes and found pigmentation to be consistent with the level of predation risk based on the principles of trophic cascades. Unfortunately, the blue-holes investigated, and were selected *a priori*, differed in taxonomic groups of copepods so it may be an influencing factor in the results, yet as pigmentation has been identified as beneficial in both groups this is unlikely. In a laboratory experiment, I attempted to determine if pigmentation also represents a plastic trait. I found the removal of UVR exposure caused the pigmentation to decrease in populations that had originally higher levels of pigmentation and the increase in the population that have suppressed pigmentation due to predation risk (**Paper IV**). Changes in predation risk however, showed minimal idiosyncratic responses in pigmentation plasticity. Despite the UVR being present year-round, unlike at high-latitudes, there was still substantial variation in the intensity, however predation pressure is believed to be constant year-round. This suggests that copepods from low-latitudes may have evolved plasticity in pigmentation related traits toward the threats that are most variable.

Conclusions

In this thesis, I have demonstrated the capability of zooplankton to assess multiple threats that vary both spatially and temporally and respond accordingly. More specifically, I have shown how zooplankton rely heavily on behaviour as an adaptive response to both biotic and abiotic threats. In **Paper I**, I showed that the social context influences the space use of females, eliciting a response similar to that of predator avoidance. In a broader context the results of this experiment suggest the sexual segregation may occur in natural population of *Daphnia* and this could affect ecosystem functioning. In **Paper II**, I demonstrated that *Daphnia* perform something akin to cost-benefit analyses in the presence of high-density foraging opportunities and a chronic stressor, resulting in weaker population level responses to UVR. **Paper III** highlighted that the delivery of a stressor, i.e., how variable the threat is, has fitness consequences. As mentioned within **Paper III** there were genotype specific effects, and while there was only limited replication at the genotypic level it does highlight the potential for local adaptation. The finding of fitness consequences towards variable environments emphasises the importance of environmental variability and the need for strategies that can minimise such cost. This notion is echoed in **Paper IV** where I have shown pigmentation profiles are consistent with the prevailing risk but plasticity is only retained towards the most variable threat. Taken together, my thesis illustrates how zooplankton balance risk and opportunity with a complimentary suite of traits and how environmental heterogeneity fuels individuality, population ecology and ultimately the evolution of biodiversity (West-Eberhard 2003, Pigliucci et al. 2006, Vogt et al. 2008).

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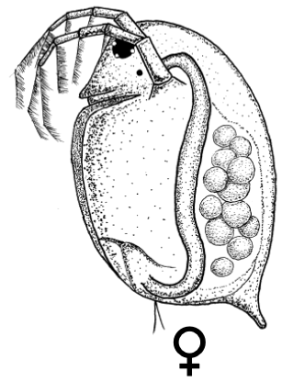
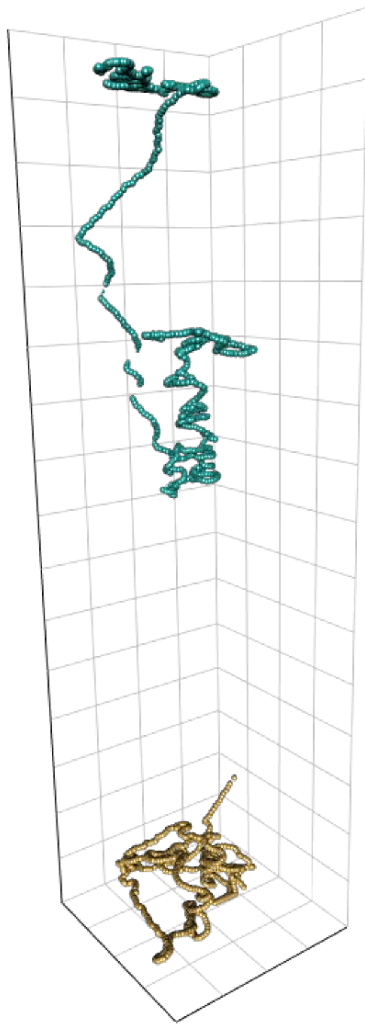
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Fear of sex: sexual conflict exposed as avoidance in a parthenogenetic invertebrate

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Abstract

Males and females often have divergent evolutionary interests, generating sexual conflicts. This is particularly true in organisms that exhibit facultative sexuality, whereby females are capable of reproducing without fitness costs of mating. Here, we provide the first documented evidence with quantitative tracking showing that sex interacts with social context to determine space-use of females, in a pattern resembling predator avoidance. To achieve this, we labelled *Daphnia magna* with fluorescent nanoparticles and utilized a 3-D tracking platform to record pairs of individuals swimming. The recordings comprised either same-sex or opposite-sex pairings. We found that females swam faster, deeper, more horizontally, and more linearly when exposed to males than when exposed to females. Simultaneously, we found that male behavior did not differ depending on swimming partner and, importantly, we observed no sexual dimorphism in swimming behaviors when swimming with the same sex. Our results suggest that the presence of males in a population has the potential to influence the distribution of individuals, similarly to known threats, such as predation. This highlights that sexual conflict has clear spatial consequences and should be considered in such ecological frameworks, like the Landscape of Fear (LOF) concept. In a broader context, the connection of the evolutionary and social concept of sexual conflict and the ecological concept of LOF may improve our understanding of population dynamics and the spatial and temporal distribution of individuals in natural ecosystems.

Significance statement

Despite the wealth of studies that detail how predators affect their prey's spatial behaviors, studies on the role of sex and social context on spatial behavior are rare. Addressing this dearth of information, we studied the swimming behaviors of an organism that can reproduce with or without sex, when exposed to an individual of either the same or opposite sex. We found no difference between the sexes in swimming behaviors; however, we revealed that females avoided males by swimming deeper in the water column, reminiscent of the response to predation. Our results highlight that social conflict between the sexes strongly affects the demographics of a population and may therefore have a substantial role in the spatial ecology of organisms in the wild.

Keywords Spatial ecology · *Daphnia magna* · Landscape of Fear (LOF) · Predation risk · Intersexual conflict

Introduction

A central challenge in ecological research is to understand the mechanisms behind differences in inter- and intraspecific spatial and temporal distributions of organisms. Interspecific

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differences are predominantly caused by competitive interactions or governed by predator distribution and behavior. For example, elk (*Cervus elaphus*) move from grassland to forested areas due to the presence of wolves (*Canis lupus*), or dugongs (*Dugong dugon*) utilize deeper waters to avoid predation from shallow water inhabiting tiger sharks (*Galeocerdo cuvier*) (Wirsing and Ripple 2011). With respect to intraspecific differences in distribution, sexual segregation is well-documented across many different taxa (Ludynia et al. 2013; Wang et al. 2018; Zak et al. 2020). Various causal mechanisms have been proposed, such as dimorphism creating different nutritional requirements, thereby segregating males and females outside the breeding season (Li et al. 2017), or avoidance of male harassment by females that have already mated (Ide 2011). Therefore, a general pattern may be that the intraspecific distribution of individuals might differ as a result of sexual conflict.

All taxa that reproduce sexually are likely to encounter some form of sexual conflict (Parker 1979), suggesting that the outcome of male–female interactions has divergent evolutionary optima for the two sexes (Chapman et al. 2003). Due to the impact of sexually antagonistic evolution, sexual conflict has been espoused as a major mechanism of evolution (Hosken and Snook 2005), with the ultimate consequences being the potential to lead to divergence within and between species (Parker and Partridge 1998; Gavrillets et al. 2001; Chapman 2018; Janicke et al. 2018). Such conflicts between the sexes can occur over various traits, including mating frequency, fertilization, and clutch size (Chapman et al. 2003). Generally, it is assumed that males benefit from maximizing such traits like mating frequency; however, females should favor lower mating rates due to the costs of sex (Gavrillets et al. 2001). These costs arise from many sources such as increased infection rates from contact with conspecifics (Thrall et al. 2000), fitness reducing seminal fluid accessory gland proteins introduced during copulation (Wigby and Chapman 2005), physical damage due to traumatic insemination (Stutt and Siva-Jothy 2001) or due to penile structures that prevent females escaping during copulation (Lange et al. 2013), increased energy demands (Nicol et al. 2019), and even increased predation risk (Magnhagen 1991).

One strategy females may employ to minimize such costs of mating is to avoid superfluous copulations such as if already mated. This is hypothesized to be more prevalent when the cost of mating is high and, therefore, females are more likely to be selective (Bleu et al. 2012). In most populations, irrespective of the fitness costs of mating, the requirement to mate in order to achieve any “fitness” may dampen the strength of these avoidance behaviors. In facultatively sexual populations however, females are potentially decoupled from the obligation to mate due to being able to reproduce asexually (Brewer 1998; Gerber and Kokko

2016). This may then lead to strong behavioral avoidance of males due to the potentially strong costs associated with mating. In such populations, it has been colorfully stated that the sequence of events in male behavior during reproductive attempts is fundamentally indistinguishable from predation attempts (Gerritsen and Strickler 1977; Brewer 1998). If true, this suggests that the male poses a potential risk to fitness for the female and consequently, the demographics of a population may have a substantial role in the spatial ecology of the population.

Daphnia magna is a common facultatively sexual freshwater cladoceran. Predominantly, *Daphnia* reproduce asexually but under certain suboptimal environmental conditions, such as at high population densities, they often switch to a sexual reproductive phase (Kleiven et al. 1992; Haltiner et al. 2020). This switch means that they produce a maximum of 2 genetically non-identical eggs through recombination, instead of bearing an asexual clutch of up to 110 live clones (Gerber et al. 2018). Change in reproductive mode is not a one-way street, and females can continue to alternate strategies between broods. Therefore, females may be under strong pressure to avoid potentially costly mating events if in the asexual phase or already mated, and could conceivably adopt different swimming behaviors as a proactive avoidance measure. Multiple studies have investigated how swimming behaviors differ between sexes of many cladocerans (*D. pulicaria* (Brewer 1998); *D. obtusa* (La et al. 2014); *Polyphemus pediculus* (Butorina 2000); *Chydorus sphaericus* (Van Damme and Dumont 2006)); however, most studies investigate interactions between the sexes, and therefore focus on group behavior or the mating behavior in high density environments. To the best of our knowledge, no studies have investigated how changes in the sex ratios affect an individual’s swimming behavior, leading to that our understanding of sexual conflicts in a spatial context is still elusive.

Hence, the purpose of our study is to disentangle the individual behavioral responses of *D. magna* in the presence of conspecifics. We hypothesize that the potential reduction in fitness due to sexual reproduction will cause females to display avoidance behaviors when paired with males. Using 3-D tracking of individual animals, we were able to quantify the speed at which males and females swim, their average swimming depth, and the tortuosity (or the linearity) of their swimming paths, i.e., we were able to map the individual behavior in different social contexts.

Specifically, we expect that due to the sexual dimorphism in size (Mitchell 2001), females will swim faster than males, and when swimming with the opposite sex, this speed will increase. Similarly, due to depth serving as a refuge from many threats, such as predation and ultraviolet radiation (UVR) (Hansson and Hylander 2009b; Ekvall et al. 2015, 2020), we predict that females exposed to males will swim deeper in the water column than either males or females

swimming with females. Multiple observations of zooplankton have described male “scanning” behavior which involves males swimming more horizontally than vertically in a bid to increase encounter chance with a female (Gerritsen 1980; Brewer 1998). Therefore, we predict that males will swim more linearly and more horizontally than females, with this effect being more pronounced when swimming with the opposite sex. In short, using 3-D tracking, we are able to provide the first insights into how sexual conflict interacts with an individual’s social context, thereby causing spatial variation in swimming behavior.

Methodology

Culture conditions

D. magna used in this experiment were isolated from a laboratory culture on the 12 August 2019, which was originally inoculated with several genotypes from a population in Lake Bysjön (55.6753 lat, 13.5452 long) in southern Sweden. They were maintained at high densities in a 400-L plastic mesocosm at 20 °C with a 16 h:8 h light:dark photoperiod and routinely fed with a predominantly *Tetradesmus obliquus* (formerly *Scenedesmus obliquus*) algal suspension. Once isolated, *D. magna* were sexed using a stereomicroscope (Olympus SZX7, Japan). Males were identified by three characteristic morphological traits: (1) the smaller and rounder rostrum in comparison to females, (2) the elongated and motile antennules, and (3) the pronounced hook at the end of the first leg (Mitchell 2001). Females were identified by the absence of these traits. The sex ratio of the initial population was approximately 10:1 (female:male). After being sexed, *D. magna* were maintained in single sex populations of 30 individuals per liter at the same temperature and photoperiod as the source population and fed with the same algal suspension ad libitum until being assayed. The behavioral assays took place over the period of 21–23 August 2019, that is, individuals were only isolated within their own sex for between 9 and 11 days; therefore, later exposure to the opposite sex was not a novel experience.

Experimental design

In order to determine swimming behaviors of individual *Daphnia*, we used a proven protocol (Ekvall et al. 2013; Palmér et al. 2016; Langer et al. 2019). This required labelling each individual with either red or yellow nanoparticles (Qdot™ ITK™ Carboxyl Quantum Dots; Life Technologies Corporation, USA) that fluoresce when excited by blue light (465 nm). This allowed us to identify both recorded individuals simultaneously. The labelling process involved binding the quantum dots to the carapace of the organism

by incubating individual *Daphnia* in 2-ml centrifuge tubes with 250- μ l aged tap water and 33- μ l quantum dot labelling solution for 1 h in the absence of light, before removing the excess solution by rinsing the organism with aged tap water (Langer et al. 2019). Once the *Daphnia* were labelled, they were recorded in a Plexiglas aquarium (0.2 \times 0.2 \times 0.75 m; filled with only aged tap water) with four cameras (Pike F-210C color cameras, Allied Vision Technologies GmbH) positioned as vertical stacked stereopairs towards the aquarium to allow 3-dimensional positioning to be acquired, and the only light source was a lighting array above supplying blue light-emitting diodes with peak emission at 465 nm (VANQ Technology). The surface light intensity was 223.4 μ mol m⁻² s⁻¹ at the top and 78.2 μ mol m⁻² s⁻¹ at the bottom (see Langer et al. 2019) which has previously been equated to a night-like condition (Ekvall et al. 2020). To discern the effects of conspecific sex on an individual’s swimming behavior, the *Daphnia* were recorded in pairs. This produced three sex combinations or “treatments”: females recorded with females ($n = 19$), males with males ($n = 20$) and females with males ($n = 22$). The pairs of individuals were obtained from separate holding aquaria (see above), and introduced to the tracking aquarium simultaneously. They were given 1 min of acclimatization before the 3-min recording began. This setup allowed us to extract multiple metrics of swimming behavior, such as the individual’s speed, depth, horizontal direction changes (horizontal net displacement ratio [HNDR]), and the tortuosity of their swimming path (net gross displacement ratio [NGDR]), as well as calculating the distance between individuals. The water was then replaced between trials to prevent lingering chemical cues influencing subsequent recordings. Due to the nature of the experiment, blinding was not possible.

Data handling and statistical analysis

We used a custom-built MATLAB application (Palmér et al. 2016; The MathWorks, Inc. 2017) to extract the *Daphnia*’s 3D positions from the recordings. Using the 3D coordinates, depth was extracted as Z coordinates, and speed was calculated as the gross distance travelled every second. HNDR was calculated as the ratio of horizontal distance travelled to the gross distance travelled every second. Similarly, the NGDR was calculated as the ratio of net distance travelled to the gross distance travelled every second. Therefore, for either ratio, the more vertical or indirect the swimming path, the lower the ratios, and conversely, the more horizontal or linear the path, the higher the HNDR and NGDR, respectively. Due to the recording frame rate (6 fps) producing between 360 and 1080 points per variable, all variables were averaged using the median values as to limit the influence of extreme values. Henceforth, all “averages” discussed refer to the median. Statistical analysis was subsequently

conducted in R version R v3.6.2 (R Core Team 2019). Figures were also drawn with R v3.6.2 utilizing the package “ggplot2” (Wickham 2016). The data and code for this study are archived online (Lee et al. 2021a, b).

To examine the effects of sex and conspecific sex on speed, we performed a linear mixed model using the package “nlme” (Pinheiro et al. 2021). Average speed served as the dependent variable, and “sex,” “conspecific sex,” and their interaction were used as fixed effects with the recording ID serving as a random effect to account for the non-independence of recording individuals in pairs. Size was also included as a covariate for the analysis of average speed, due to larger individuals having the potential to swim faster (Dodson and Ramcharan 1991; Hylander et al. 2014). Due to the physical constraints of the experimental arena, we treated the average depth as a ratio of vertical position for the analysis, i.e., occupied depth in relation to of the total available depth. As depth, horizontal movements (HNDR), and tortuosity (NGDR) variables represent ratios derived from continuous numbers, we employed beta regression mixed models (Douma and Weedon 2019). “Sex,” “conspecific sex,” and their interactions were also used in these models as fixed effects with the recording ID as a random effect. All models were graphically investigated, and the examination of significant differences in main effects was conducted using the post hoc Tukey’s test. In the case of the dependent variable depth, we note that the model appears over-dispersed; however, other forms of modelling provide less accuracy due to the methodological design. To provide easier interpretation of the results, we back transformed the depth variable to show actual depth as opposed to a ratio of vertical position.

Results

Speed

Swimming speed was, as expected, influenced by size with larger individuals swimming faster than smaller ones (Table 1; online resource Fig. S1.). Yet, when accounting for size, we found no single effect of sex on swimming speed. There was, however, an effect of swimming partner on the speed, as different sex pairs show distinct speeds, while same-sex pairs show no difference. Importantly, we

observed a strong interaction between sex and the swimming partner. This effect was entirely driven by the females swimming faster when swimming with a male than with a female (Tukey’s test; $p=0.003$). Specifically, females from same-sex pairs swam approximately 32% slower than females swimming with the opposite sex (Fig. 1a). Males did not adjust swimming speed with different swimming partners (Tukey’s Test; $p=0.93$) swimming at 13.81 mm s^{-1} (± 1.14 SE) with females and 13.05 mm s^{-1} (± 1.08 SE) with other males.

Depth

Swimming partner also influenced the focal individual’s swimming depth whereby same-sex pairs swam higher in the water column than opposite sex pairs (Table 2). There was also evidence that males preferred swimming higher in the water column than females. Similar to speed, there was a clear interaction effect of sex and swimming partners with females having an estimated mean swimming depth of 586 mm (± 38 SE) when swimming with a male, whereas swimming with another female averaged 379 mm (± 39 SE) (Tukey’s test; $p < 0.001$), that is, females swam 64% deeper in the presence of a male than of a female (Fig. 1b). Males however had similar depth preferences irrespective of the swimming partner’s sex (Tukey’s Test; $p=0.97$).

HNDR

Horizontal movements (HNDR) did not conform to prior expectations, since males swimming with females were less likely to perform horizontal movements than their female counterparts (Fig. 1c). The sex of the swimming partner also had a notable impact on the ratio of horizontal movements, with females in same-sex pairs being less likely to perform horizontal movements than with the opposite sex. The interaction of sex and swimming partner sex also emerged as significant. This appears to be driven by females paired with males, as they were 15% more likely to swim horizontally than their male counterparts, or 11% more likely to swim horizontally than females exposed to a female (Tukey’s tests; $p < 0.001$ and $p = 0.004$ respectively). Males in comparison did not differ in HNDR when exposed to a female or to a male (Tukey’s test; $p=0.99$).

Table 1 Linear mixed-effects model results with recording ID as the random effect using the lme function to account for the heterogeneity in variance among groups

	Fixed effects	Estimate	s.e	d.f	<i>t</i> value	Pr (> t)
Average speed	Size	7.72	3.14	59	2.461	0.017
	Sex	-0.84	1.91	59	-0.443	0.66
	Conspecific sex	-4.67	1.27	58	-3.684	<0.001
	Sex × conspecific sex	3.91	1.71	58	2.289	0.026

Fig. 1 Effects of sex and conspecific sex on various metrics of swimming behavior in *Daphnia magna*. The subplots refer to (a) average speed considering size as a covariate, (b) average depth, (c) average ratio of horizontal to vertical movements (HNDR) with 1 being completely horizontal and 0 being completely vertical, and (d) the average net to gross displacement ratio (NGDR) with higher values indicating a lower turning rate. All subplots show the model estimates ± 2 SE with raw values (individuals) plotted as faded points. The dashed lines in subplot (b) represent the boundaries of the water column

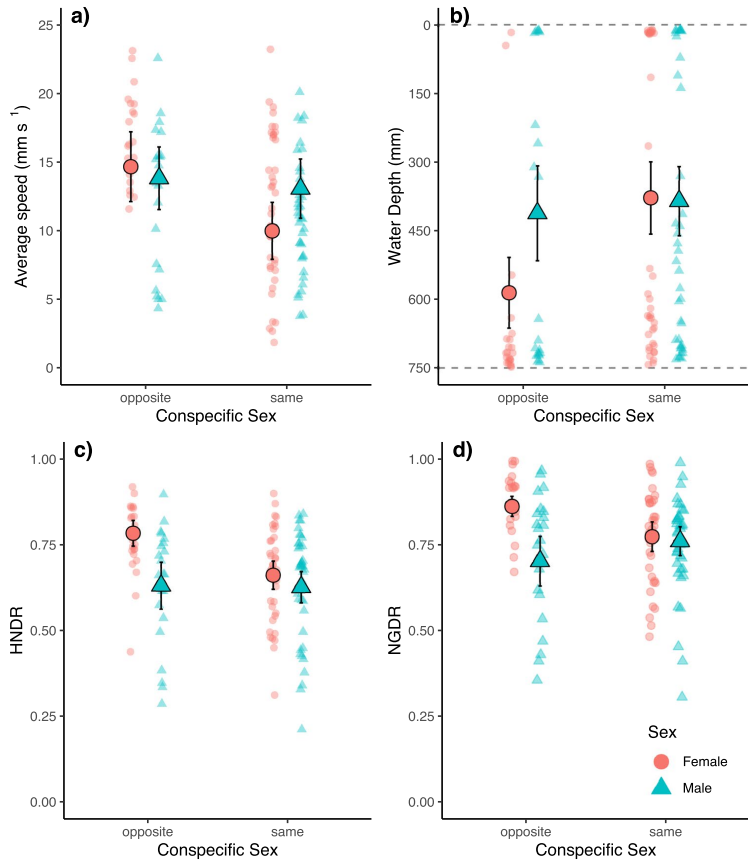


Table 2 Results from generalized linear mixed-effects beta regression models with variable ϕ

	Fixed effects	Estimate	s.e	d.f	z value	Pr (> z)
Average depth	Sex	-1.073	0.411	113	-2.610	0.009
	Conspecific sex	-1.252	0.368	113	-3.406	<0.001
	Sex \times conspecific sex	1.110	0.503	113	2.205	0.027
Average HNDR	Sex	-0.717	0.171	115	-4.185	<0.001
	Conspecific sex	-0.586	0.167	115	-3.514	<0.001
	Sex \times conspecific sex	0.556	0.217	115	2.558	0.011
Average NGDR	Sex	-0.819	0.197	115	-4.168	<0.001
	Conspecific sex	-0.462	0.192	115	-2.411	0.016
	Sex \times conspecific sex	0.710	0.249	115	2.854	0.004

All models used the recording ID as a random effect

NGDR

The tortuosity (NGDR) of an individual’s swimming path showed considerable influence of all main fixed effects.

Female *Daphnia* exposed to a male were more likely to have linear swimming paths than their male partners (Fig. 1d), and female same-sex pairs were also more likely to swim more linearly than females with males. The interaction

between both sex and the sex of the swimming partner yielded an effect, which once again was solely driven by females paired with males. Females were approximately 15% more likely to swim linearly than their male counterparts (Tukey's test; $p < 0.001$).

Discussion

Sexual conflict is a widespread social phenomenon among sexually reproducing organisms with the potential to shape macroevolutionary patterns (Bonduriansky 2011). Despite the prevalence of obligately sexual organisms, there are numerous examples of alternative reproduction strategies, including facultative sexuality (Burke and Bonduriansky 2017; Kobayashi 2019) among, for example, numerous common and globally distributed organisms, such as many crustacean taxa. Here, we show that facultatively sexual *D. magna* females modify their behavior when exposed to males, swimming faster, deeper, more horizontally, and straighter than when exposed to other females. In contrast, males do not alter swimming behavior depending on their swimming partners' sex. This finding suggests that the costs of sexual reproduction for females trigger their avoidance of conspecific males.

Past studies of the genus *Daphnia* have frequently looked at mating behaviors, and generally focused on how males increase their probability of encountering a female (Brewer 1998), neglecting how females act in these situations. This is often a consequence of the methodological design, which requires both males and females to be present in order to observe potential mating events. However, in nature, there are often times when males will be absent and curiously, few studies use single sex populations as controls, and yet extract and discuss the behavior of females (Brewer 1998). Here, we provide this missing information and observe single sex and opposite sex pairings.

In several studies, it has been discussed that *D. pulex* males swim twice as fast as females in a bid to increase encounter rates via "scanning" behaviors similar to other zooplankton taxa (Brewer 1998). The scanning behaviors are characterized by more horizontal and linear movements (Gerritsen 1980), and have even been suggested to be widespread in the planktonic community as they are found in copepods from both marine and freshwater environments, as well as in *Daphnia* species (Gerritsen 1980; La et al. 2014). Intuitively, this appears logical as, if males can exploit a single plane with relatively straight movements, it increases the probability of encountering randomly distributed resources, such as females (Dusenbery 1992; Visser 2007). *D. magna*, however, do not appear to echo this pattern as, when accounting for size, we show here that males do not swim faster than females, and only females alter their

speed according to their swimming partners. Moreover, we observed that females appear more likely to perform more horizontal and straighter movements when exposed to males, although we cannot exclude that this may be an artefact since females cannot swim further in the vertical plane when reaching the bottom and were therefore forced to swim more horizontally. Despite this, males do not appear to differ from females when in same-sex pairings nor when paired with a female, which supports our view that this "scanning" behavior does not occur in *D. magna* and calls into question whether this is indeed a widespread behavior in zooplankton (Brewer 1998). Instead, our results give credence to the notion that males' likelihood of reproducing relies heavily on chance encounters with sexually responsive females (Kawatsu 2013; Gerber and Kokko 2016).

Despite the lack of similarity in the previous swimming behaviors recorded with other species, the use of depth as a refuge is well described for *D. magna*. They use deeper waters to attenuate harmful ultraviolet radiation (UVR) and avoid predation from visually hunting predators, such as fish (Hansson and Hylander 2009b). Similarly, our data show that female *Daphnia* respond to males in a pattern resembling a threat response (online resource Fig. S2), i.e., the female avoids the male by diving deeper. Despite the clear overall response, not all females resided deeper in the water column. This variation in optimal depth may be a result of sexual receptivity, although we cannot rule out other factors such as genetic variation or energetic state which likely contribute to this trait. Interestingly, males did not display a propensity to follow the majority of females to the deeper waters, which strengthens findings from previous studies showing males to only be able to follow females for a few body lengths (Brewer 1998); however, to what degree this is male sensory limitations or choice in pursuit is unknown. Furthermore, we did not observe any explicit following of partners in any treatment group when looking at the distance between individuals (data not shown).

The consequences of the observed avoidance may have far-reaching effects on the population dynamics. For instance, the energetic cost of performing avoidance behaviors has been demonstrated to reduce population growth in a facultatively sexual invertebrate (Nelson 2007). Also, male *Daphnia* in particular have been shown to be more positively phototactic than females (De Meester 1993), which suggests they inhabit higher strata than females, which may thereby explain the female use of depth as a refuge, as shown in our study. However, for the females inhabiting deeper waters, there are further potential metabolic costs. For example, if in a sufficiently deep lake, the temperature gradient in the deeper waters may reduce metabolic rates (Dawidowicz and Loose 1992), coupled with lower food availability and quality, which suggests that population growth would be retarded. That said, it is well established that *D. magna*

perform diel vertical migration (DVM) as a foraging strategy to avoid the higher predation risk and UVR during the day, foraging in the food-rich surface waters during the night (Hansson and Hylander 2009a, b). This strategy could mitigate some of the potential costs of males inhabiting the higher strata, especially if mating success is increased with light availability. Under this scenario, our results suggest that sexual conflict could be a further selective pressure contributing to the evolution of this DVM behavior.

In accordance with our findings, the sequence of events by males during reproductive attempts has previously been mentioned as being indistinguishable from predation attempts (Gerritsen and Strickler 1977; Brewer 1998). Predator–prey interactions in a spatially explicit context have been extensively studied (Miller et al. 2014; Fortin et al. 2015; Schmitz et al. 2017), whereas the spatial ecology of sexual conflict has received relatively little attention. An emergent framework within the predator–prey domain aimed at clarifying and refocusing the effort to understand the spatial effects of risk is the “Landscape of Fear” (LOF) (Laundré et al. 2001, 2010, 2014; Brown and Kotler 2004; Gaynor et al. 2019). The LOF has been defined as the spatial variation in prey perception of predation risk. In order to proactively minimize such risks, the LOF concept suggests that two behavioral strategies may be employed, and they are (1) avoiding areas of high predation risk and (2) modifying behavior in a location to reduce the probability of predation (Gaynor et al. 2019). Replacing the word “predation” with “mating,” we see that *Daphnia* do indeed avoid areas of high risk, i.e., where males are located. Therefore, based on the potential for sexual conflict in this facultatively sexual species (Gerber and Kokko 2016), we suggest that wherever there are probable fitness costs, this framework could be applied. Our results highlight that demographic features such as reproductive mode or sex ratios which vary over the season may be an important factor in the perception of risk for female individuals and is an avenue for further research.

In conclusion, we observe here that males and females of *D. magna* lack sexual dimorphism in swimming behaviors. However, when in the presence of the opposite sex, females demonstrate behaviors consistent with strong male avoidance, leading to a skewed depth distribution among sexes. These avoidance behaviors are analogous to other threat responses, such as to predation risk or ultraviolet radiation, which have been shown to have fitness consequences. Therefore, we advocate that incorporating predominantly evolutionary concepts, such as sexual conflict, to the ecological frameworks, like the Landscape of Fear, has the potential to improve our understanding of the mechanisms determining the distributions of individuals in space and time.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00265-021-03054-9>.

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Author contribution All authors conceived the project and contributed to writing. CSU and ML collected data. ML performed the data analysis and wrote the first version of the manuscript.

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Data availability Data supporting the results are available online in the Dryad data repository at <https://doi.org/10.5061/dryad.2ngf1vhm8>.

Code availability The code used to analyze the data is available online in the Zenodo repository at <http://doi.org/10.5281/zenodo.5112715>.

Declarations

Ethics approval All animal handling and husbandry was conducted in accordance with approved institutional guidelines. The license M182-15 was granted by the Malmö/Lund authority for ethics of animal experimentation.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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Supplementary Information

Fear of sex: Sexual conflict exposed as avoidance in a parthenogenetic invertebrate

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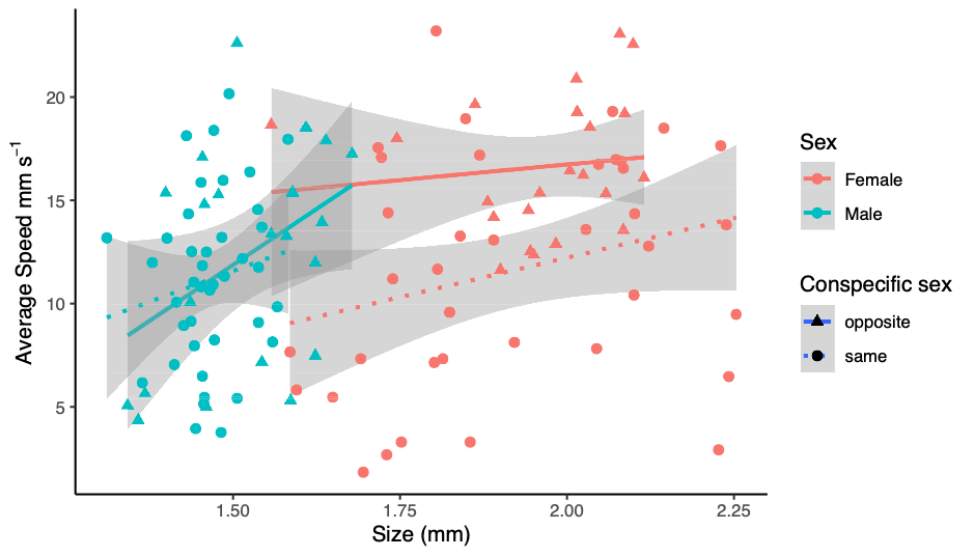


Figure S1. Swimming speed plotted against body length. Males swim equally as fast irrespective of the conspecific's sex, whereas females swim faster in the presence of a male than a female. The sexual dimorphism in size is very apparent, however both sexes have the potential to swim at approximately the same absolute speeds, which implies the smaller males are relatively faster than the larger females.

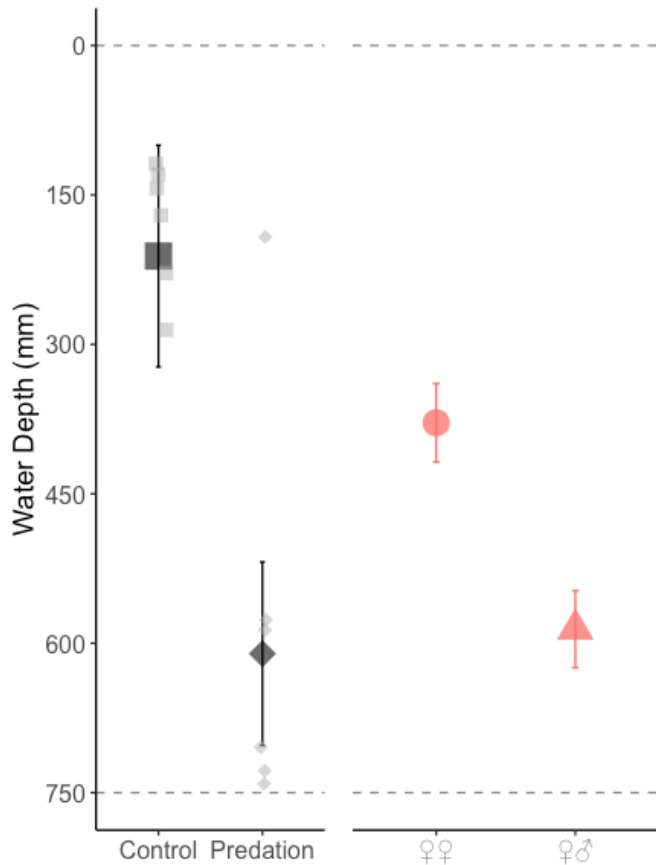


Figure S2. Swimming depth of females when not exposed (control) or exposed to a predator (black symbols) alongside the results of when females are exposed to either another female or male (circle and triangle symbols, respectively). Large points represent the model estimates ± 2 SE with the raw data of the predation risk experiment plotted as small grey points ($n = 6$).

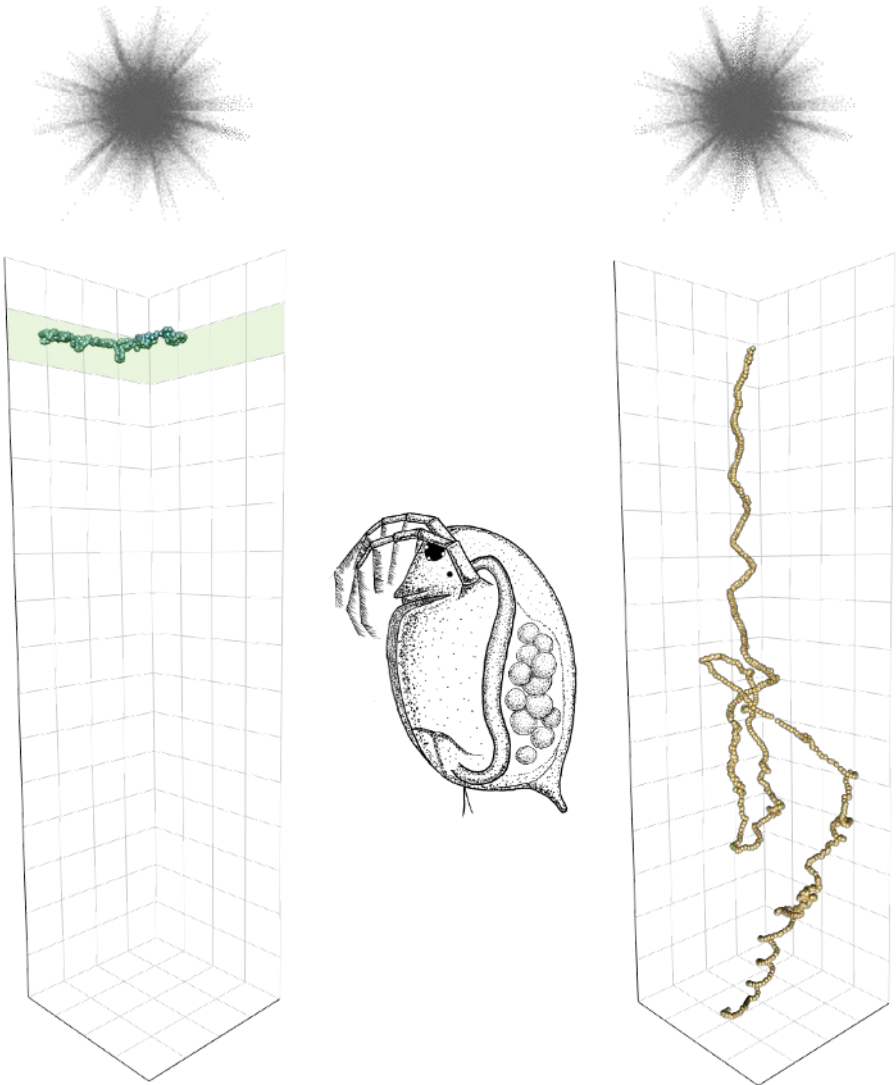
Predation experiment methodology

To demonstrate how predation affects spatial distribution of *Daphnia magna* in the same experimental set up, detailed in the main article, we performed a separate experiment based on chemical risk cues. We generated such cues using crucian carp (*Carassius carassius*), a fish predator of *D. magna*. Under the ethical permit M182-15, six wild-caught juvenile crucian carp were obtained from a pond in Lund, Sweden, and maintained in a 152 L aquarium at 16 °C with a 16h : 8h light : dark photoperiod. The carp were routinely fed with both frozen bloodworms and *Daphnia*. After 4 weeks under these conditions, 6 L of water was removed to the experiment and the fish were released at the site of capture. The water sample was filtered through a 20 μ m mesh to

remove any large particles yet retain fish kairomones and alarm cues. To ensure the presence of chemical cues, 48 hours prior to water collection the carp were also fed with approximately 500 live *Daphnia*. The water was then split into 1 L samples and frozen. Each frozen sample was left to thaw at 20 °C for 12 hours before the experiment.

To generate the predation risk treatment, one sample of predator cues was mixed with 24 L of aerated tap water and the control treatment group comprised 25 L of aerated tap water. The design of this experiment followed the procedure detailed in the main body of the text. That is to say, individual *D. magna* were labelled with fluorescent nanoparticles, introduced to the aquaria, which was either ‘predation’ or ‘control’, and recorded for 3 minutes. Due to the nature of the treatments, each individual was tracked in isolation and with no acclimation period.

Statistical analysis was conducted in R v3.6.2 after extracting the 3D positions in the same manner as in the main text. This meant fitting a beta regression model, with one fixed effect ‘Treatment’. It was evident that predation risk caused individuals to alter their swimming behavior. Specifically, predation risk exposed *Daphnia* swam approximately 3 times deeper compared with control individuals ($\beta = 2.41$, SE = 0.57, $z(9) = 4.24$, $p < 0.001$).



Daphnia magna trade-off safety from UV radiation for food

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Abstract

Research on diel vertical migration (DVM) is generally conducted at the population level, whereas few studies have focused on how individual animals behaviourally respond to threats when also having access to foraging opportunities. We utilized a 3-D tracking platform to record the swimming behaviour of *Daphnia magna* exposed to ultraviolet radiation (UVR) in the presence or absence of a food patch. We analysed the vertical position of individuals before and during UVR exposure and found that the presence of food reduced the average swimming depth during both sections of the trial. Since UVR is a strong driver of zooplankton behaviour, our results highlight that biotic factors, such as food patches, have profound effects on both the amplitude and the frequency of avoidance behaviour. In a broader context, the trade-off between threats and food adds to our understanding of the strength and variance of behavioural responses to threats, including DVM.

Keywords: Behaviour, 3D tracking, UV Threat, Zooplankton, Foraging, Vertical position

Introduction

Energy may be considered the currency with which all life is concerned. However, the acquisition of energy is far from simple in the complex landscapes that all organisms live. Foraging is a risky activity as during the search for food, individuals are more often exposed to threats. Therefore, foraging should occur only until the benefits are equal to the costs of further foraging (Brown and Kotler 2004). The decisions whether to forage or avoid a threat, e.g. predation, have been both theoretically (Brown and Kotler 2004) and empirically studied in countless taxa, such as invertebrates (Kohler and McPeck 1989), amphibians (Eklöv and Halvarsson 2000), birds (Olsson et al. 2002) and mammals (Brown and Kotler 2004).

One of the largest foraging movements, in terms of biomass, is diel vertical migration of zooplankton (DVM), which is unique to aquatic environments (Lampert 1989, Hays 2003). This is the large scale and daily movement from surface waters during the night to the deep waters during sunlight hours, occurring in both marine and freshwater systems. Both the proximate and ultimate mechanisms of this movement have been investigated for decades with a prominent explanation being food-rich surface waters serving as an opportunity, coupled with increased predation risk from visually hunting predators acting as a threat (Lampert 1989, Bandara et al. 2021). However, DVM has been documented from environments where such predation was not a strong predictor, suggesting that other factors are important in the cost benefit analysis when foraging (Muluk and Beklioglu 2005). Under these scenarios, solar UV radiation (UVR), which is the high-energy wavelengths between 280-400 nm (Tucker and Williamson 2011), is often considered an important environmental driver of this foraging migration.

UVR is an appreciable risk in aquatic environments (Hansson and Hylander 2009), with considerable negative effects being reported at many trophic levels (Peng et al. 2017). With regards to zooplankton, UVR has been shown to reduce survival (Rautio and Korhola 2002), impede growth and decrease fecundity (Grad et al. 2001), as well as inducing behavioural avoidances (Hansson et al. 2016, Heuschele et al. 2017). Despite the plethora of negative effects, UVR is attenuated over depth providing a refuge deeper in the water column and therefore penetration of UVR is highly variable between lakes, penetrating between 0.5 m and +20 m varying with the lake transparency (Tucker and Williamson 2011). Particularly clear lake communities, such as alpine lakes, are far more susceptible to the effects of UVR than communities in lakes

high in coloured dissolved organic matter, which has been demonstrated to affect behaviour of zooplankton (Wolf and Heuschele 2018). Along with variation between lakes, the rapid changes in cloud cover create acute temporal variation that demands equally instant changes in response to UVR fluctuations (Hansson et al. 2016).

Certain concepts such as ‘ideal free distribution’ which consider resource availability have been very successful in explaining the vertical distribution of zooplankton under predation risk and other abiotic variables (Maszczyk et al. 2018, Maszczyk et al. 2021). However, UVR has not specifically been investigated in such models, in spite of the confirmation of UVR being an important driver in the spatial positioning of zooplankton through field observations or population level experiments (Leech and Williamson 2001, Rose et al. 2012). Therefore, the objective of this study was to investigate the mechanistic role of spatial heterogeneity in determining foraging decisions of a common zooplankter under risk conditions. Here we specifically focus on how individual *Daphnia magna* respond to the trade-off between remaining in the food-rich surface water or avoiding the potentially harmful UV radiation. We hypothesize that *D. magna* will have weaker response to UVR threats when in the presence of foraging opportunities, i.e., that they trade-off safety for food.

Methodology

Culture conditions

Daphnia magna were obtained from a laboratory population initially inoculated with several genotypes originating from lake Bysjön (55.6753 lat, 13.5452 long). This culture was maintained in a 12 L plastic aquarium at 20 °C with a 16 : 8h light : dark photoperiod for over 100 generations. They were fed *ad libitum* with a predominantly *Tetrademus obliquus* algal suspension. Three days prior to the behavioural assay individuals were randomly selected and transferred to pre-experimental holding jars to reduce the effects of competition. These consisted of a 100 ml jar filled with 80 ml aged tap water and individuals were fed with 120,000 cells ml⁻¹ of a single species culture of *T. obliquus* (NIVA-CHL 6) for two days before being transferred to a new jar with the same conditions for one further day. This ensured a high quality and quantity of food was available, especially immediately prior to the behavioural assay.

Behavioural assay

Each *Daphnia* was individually assayed by adapting a proven protocol for tracking the swimming behaviours of mm-sized zooplankton (Ekvall et al. 2013, Langer et al. 2019). Individuals were transferred to 2 ml centrifuge tubes and labelled with fluorescent nanoparticles (655 ITK Carboxyl quantum dot, fluorescent at 655 nm; Life Technologies, Carlsbad, California, USA, Prod. Nr.: Q21321MP). Assays were conducted in an experimental aquarium (0.2 x 0.2 x 0.75 m), upon which sits a lighting unit with 8 blue light-emitting diode (LED) arrays, acting as excitation lights for the fluorescent nanoparticles. In the centre of the lighting array there is a UVR LED (100 µW/cm²; ENFIS UNO Tag Array Ultra-Violet 375 nm UV-A ENFIS LIMITED, Swansea, United Kingdom) which simulates UVR threat. Facing the aquaria are four synchronized digital cameras (Pike F- 210C, Allied Vision Technologies GmbH, Stadroda, Germany), which allows the recording of videos and the triangulation of 3-D coordinates. Further details on the labelling process and experimental system can be found in Ekvall et al. 2013 and Langer et al. 2019.

To test swimming behaviours in the presence of a high-density food patch, *Daphnia* were either tested in the aquarium with 200 ml of *T. obliquus* culture, which was heated to 42 °C and slowly introduced to the surface of the water, or 200 ml of 24- 48h aged tap water heated to the same extent and introduced in the same manner. The temperature difference between the aquarium water and the introduced medium created a spatially explicit food patch ($n = 46$) or ‘control’ temperature patch ($n = 28$) at the surface. We performed this experiment in blocks, i.e., we used up to a maximum of 5 separate individuals sequentially in the same arena before removing all the water, cleaning the recording arena and resetting the conditions. We measured the algal concentration, temperature and ultraviolet irradiance in situ. The algal concentration was measured at 3 depths (surface, middle and bottom; 0, 25 & 75 cm respectively) using the AlgaeLabAnalyzer (bbe Moldaenke GmbH, Schwentingen, Germany) at the end of each experimental block for a conservative estimate of the spatial variation in the food patch. Temperature was measured at the beginning of each block as to ensure the starting temperature was within the thermal tolerance for *Daphnia magna* (5-30 °C) (Seefeldt and Ebert 2019). Repeated measurement of UV irradiance rapidly disperses the food patch throughout the water column, therefore this was only measured once by lowering a radiometer (IL 1400A; International Light; Newburyport, MA, USA) with sensors for UV-A (320–400 nm) through the water column, before the data collection began.

Daphnia have been suggested to sense food patches using visual, mechanical and olfactory cues, however there are conflicting reports (van Gool and Ringelberg 1998, Roozen and Lurling 2001). To ensure individuals had the same opportunity to detect the food patch, we carefully introduced each individual to the surface water of the aquarium, directly exposing them to the patch, and the assay began immediately. The behavioural recording lasted for two minutes with two distinct phases. The first minute may be considered the acclimation phase and the second was the threat phase, mimicking solar radiation, whereby the UVR LED was turned on. Due to the location of the food patch at the top of the aquarium, the positioning of the UVR LED creates a direct trade-off between foraging opportunities and threat. This threat has been repeatedly shown to elicit a response in *Daphnia magna* whilst in clear water (Hansson et al. 2016, Heuschele et al. 2017, Ekvall et al. 2020). Using MATLAB, we obtained coordinates from the tracks of the videos and using R v 3.6.2 (R Core Team 2019) we were then able to extract three-dimensional positions from the list of XYZ coordinates. Missing coordinates between two known coordinates were interpolated using the package *zoo* (Zeileis and Grothendieck 2005).

Data handling and analysis

All data handling and statistical analyses were conducted with the software R v 3.6.2. The data and code for this study are archived online (Lee and Hansson 2021). For all analyses, we used the median of each individual recorded as the ‘average’ to limit the influence of extreme values, unless otherwise specified. Since we obtained the positions during both the acclimation and UVR phase, we subsequently utilized the average vertical position in either phase as the dependent variable. We then analysed the average depth using Mann-Whitney U tests due to non-normality. We also utilized linear regression to investigate correlations between the mean swimming depth during each recording block and the environmental conditions during that behavioural assay. If UVR level alone would explain the depth distribution in the water column, we would expect a similar number of animals above and below the same UV level between both treatments. This was tested by comparing the frequency of animals at the UVR level corresponding to the depth of mean chlorophyll-a both with and without a food patch using a Pearson’s X^2 .

Results

When first entered into the recording arena, the *Daphnia* exposed to foraging opportunities remained higher in the water column than those without foraging opportunities ($U = 846$, $n_{[\text{No Opportunity}]} = 28$, $n_{[\text{Opportunity}]} = 46$, $p = .024$; Fig. 1). Similarly, when exposed to a threat, in this case UVR exposure from the surface, more individuals stayed near the surface when offered a foraging opportunity (i.e., food patch) than when no such opportunity was present ($U = 927$, $n_{[\text{No Opportunity}]} = 28$, $n_{[\text{Opportunity}]} = 46$, $p = .001$; Fig. 1). To ensure that differences recorded during UVR were not simply an artifact of autocorrelation with the acclimation phase, i.e. the phase before the UVR threat, the change in depth between the two phases was also compared and the result persisted. That is to say, when offered a food patch *Daphnia* individuals were not responding as strongly to UVR as those without feeding opportunity at the surface ($U = 318$, $n_{[\text{No Opportunity}]} = 28$, $n_{[\text{Opportunity}]} = 46$, $p < 0.001$; Fig. 2), meaning that animals swam slightly higher in the water column when the algae concentration was higher.

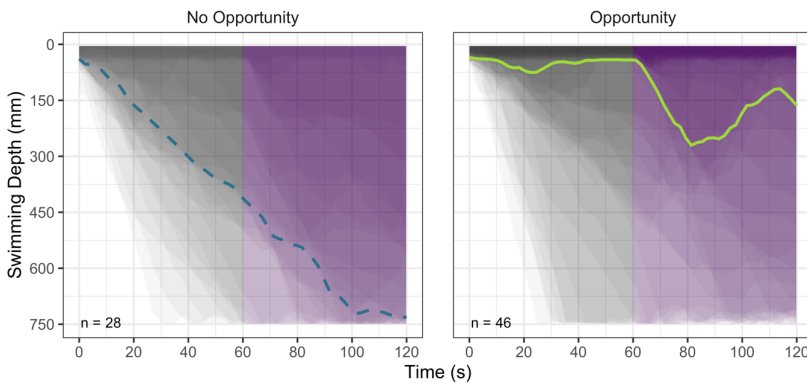


Figure 1. Individual swimming depths (areas plotted over one another) during experimental assays offering no feeding opportunity (left) and a food patch at the top (right). The median depths are indicated as colored lines, where the solid green line represents the median depth with an opportunity (food patch) and the dashed blue line shows the median without a surface feeding opportunity. The grey section (first 60 seconds) represents the acclimation phase and the purple (last 60 seconds) denotes when individuals were exposed to a UVR threat from above.

Having established that *Daphnia* can sense the algal food patch at the surface and react accordingly, that is, show less avoidance to UVR in its presence, we calculated the mean of the average swimming depth for each recording block and checked the correlation of quantity of the algae at the surface of the water. We found that the mean swimming depth of *Daphnia* was marginally negatively correlated with the concentration of algae present in the food patch before ($F_{1,16} = 4.193$, $r^2 = 0.208$, $p = 0.057$) and during the UVR exposure ($F_{1,16} = 4.494$, $r^2 = 0.219$, $p = 0.050$). To ensure that the temperature gradient, introduced to create stratification, did not influence the swimming depth we also checked the surface temperature of each experimental block and the mean swimming depth for that block and found no correlation either before ($F_{1,27} = 0.002$, $r^2 = -0.037$, $p = 0.97$) or during UVR exposure ($F_{1,27} = 0.531$, $r^2 < 0.001$, $p = 0.47$). Similarly, we tested if the food patch affected the behavioural response through stronger UVR attenuation than without a food patch. Hence, at a depth with the same UVR irradiance ($21.6 \mu\text{W}/\text{cm}^2$, which is the irradiance at the depth of mean chlorophyll; 207 mm; Fig. 3), the frequency of individuals was negatively correlated with being above this depth without foraging opportunities, whereas it was instead positively associated with being above this depth in the presence of a foraging opportunity ($\chi^2_{[1, N=74]} = 4.095$, $p < 0.05$).

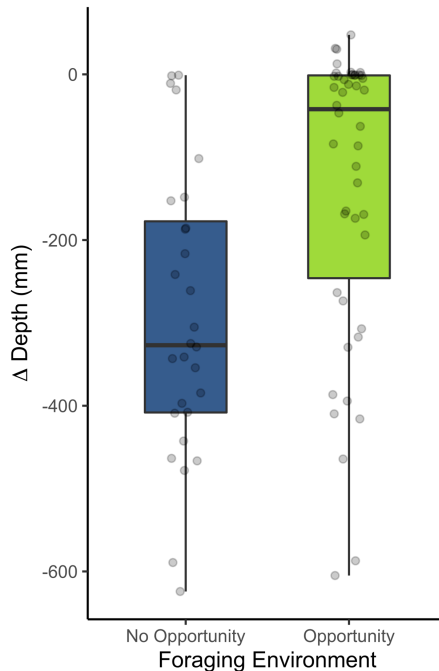


Figure 2. Change in median depth (Δ depth) from before (first 60 seconds) to during exposure to a UVR threat, showing that in the presence of food (opportunity), the response to UVR is weaker.

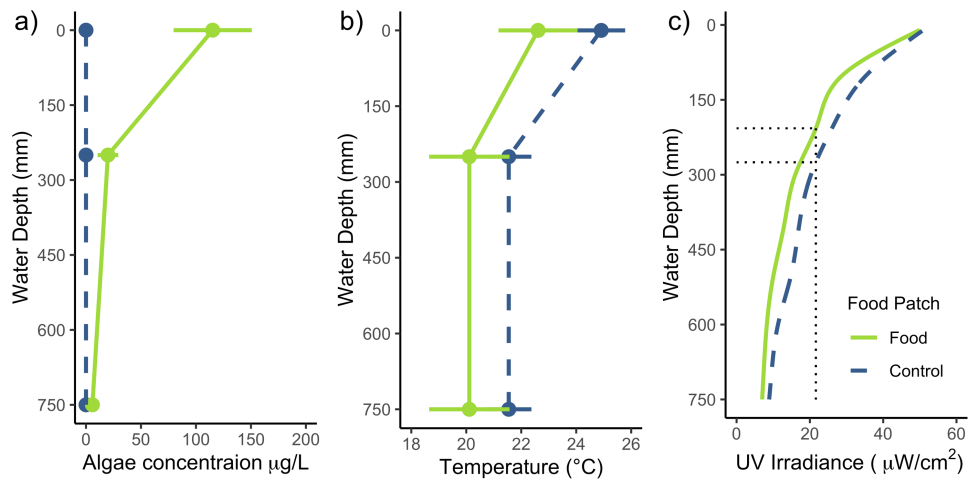


Figure 3. Physical characteristics of the experimental arenas. All figures have been rotated to most obviously display the spatial variation over depth of each parameter. Figure 3 a) displays algae concentration, b) temperature and c) the ultraviolet irradiance. Each point represents mean ± 2 SE with green lines denoting the enclosure characteristics with foraging opportunities and the dashed blue lines representing the conditions with no foraging opportunities, i.e., only a temperature patch. The dotted lines in panel c) clarifies that the difference is negligible between the two experimental conditions with regards to the depth of similar UVR threat, i.e., the algal food patch did not affect the UVR attenuation through the water and thereby unlikely to have affected the behavior of the animals.

Discussion

Foraging is a critical feature among all animals, and how they trade-off the opportunities of energetic gain versus the costs associated with risk is a central tenant of ecology (Brown and Kotler 2004). Diel vertical migration, one of the largest foraging phenomena in terms of biomass, has long been studied in relation to predation, ultraviolet radiation (UVR) and even temperature (Lampert 1989). To the best of our knowledge, there are no studies that explicitly, and at the individual level, test how the chronic stressor UVR interacts with spatial heterogeneity in opportunities for foraging. Therefore, the rationale of our study has been to advance our knowledge of individual zooplankton responses to this common and globally distributed threat, when also offered an opportunity to feed. Hence, our study mechanistically addresses how UVR and patchy food supply may affect the spatial and temporal variance in one of the largest daily biomass movements on Earth - diel vertical migration (DVM) of zooplankton. We fully appreciate that lab-based studies are often far removed from natural conditions, however we here demonstrate experimentally that a high-density food patch is indeed a strong incentive for individual *Daphnia magna* to remain in surface waters, even in the face of the substantial risks UVR exposure imposes.

Daphnia are an integral member of most freshwater ecosystems worldwide, being a dominant grazer on phytoplankton and in turn, being a food source for higher trophic levels. They have a rapid output of offspring, taking approximately 10 days until maturity and then generating broods of parthenogenetically produced offspring every 3-4 days until death (Ebert 2005). Hence, in accordance with our results and under the umbrella of the 'pace of life syndrome' concept (Réale et al. 2010), *Daphnia*, may perceive the risks associated with UVR exposure as less important than the acquisition of energy.

According to the transparency regulator hypothesis (TRH) (Tucker and Williamson 2011, Williamson et al. 2011), differences in the physical environment such as UVR and algal abundances may modulate the depth that zooplankton are found at (Williamson et al. 2011). In our experimental set-up the attenuation of UVR was negligibly affected by the food patch compared to the control environment. However, as a critical test that the reduction in UVR was not the driver of our findings, we checked the frequency of individuals above and below the UV irradiance equivalent to just below the food patch for both experimental conditions. We found a positive

association between the food patch and individuals remaining at the surface and a stronger negative association with surface waters in the control conditions. This excludes the possibility that the food patch reduced the level of UVR, thereby providing refuge. Hence, we may conclude that UVR is less important in driving DVM when there are high densities of algae in surface waters despite the same sublethal UVR levels being prevalent. Similarly, previous studies have also indicated that temperature may affect the vertical distribution of zooplankton (Dawidowicz and Loose 1992), and that they will, when not foraging, retreat to cooler waters and thereby reduce their metabolism. However, in our experiment we were unable to show that *Daphnia* distributed themselves to any thermal gradient, although we cannot discount the possibility of synergistic effects between temperature and algae.

Despite the clear statistical findings that the UVR, as well as the opportunity to feed in surface waters, affected the vertical distribution of *Daphnia*, we note that there is a high degree of inter-individual variation within each experimental condition (see for e.g. Fig. 1). This is very much in line with previous studies using the same methodology (Hansson et al. 2016, Heuschele et al. 2017), as well as the numerous reports showing the wide spatial distributions in natural ecosystems (Stich and Lampert 1981, Duffy 2010), suggesting that high variation in behavioural traits is a common and natural phenomenon under controlled conditions, as well as in the wild. There are multiple potential explanations for such commonly observed and high variances and they are not necessarily mutually exclusive. One potential explanation is related to the asset protection principle (Clark 1994), predicting that as the residual reproductive value (RRV) decreases (with age, for example), individuals will increase risk taking behaviours and, conversely, individuals with a high RRV will ‘play it safe’ (Moschilla et al. 2018). Applying the RRV concept to our experiment may expose that the intraspecific variation in behaviour is related to the energetic state of the individuals. In our study, however, we standardized the short-term energetic state through creating competition free food availability, although we cannot exclude that age, reproductive state, or energetic reserves may have differed somewhat in our experiment, thereby adding to the variance.

Interestingly, we also noted a tendency for *Daphnia* to conform to one of two strategies. It appeared as though individuals either remained near the surface or resided near the bottom, with few individuals averaging at intermediate depths. Specifically, 75% of individuals without a foraging opportunity and 76% with a foraging opportunity occupied either the top or bottom 25% of the available space. This suggests that *Daphnia* may actually have ‘personalities’ (or rather behavioural types) with some having a “bold” and others a “shy” attitude towards entering a novel environment (Heuschele et al. 2017); a phenomenon well-known among mammals and other higher animals, for example, fish (Chapman et al. 2011).

In conclusion, our results suggest substantial intraspecific variation in threat response which, in a keystone species like *Daphnia magna*, could have important consequences for communities and ecosystems (Duffy 2010). Hence, in a broader context our results suggest that the UVR avoidance behaviour, as well as the phenomenon of DVM, will be modified by *Daphnia* at the individual level depending on the food availability in surface waters, concluding that the DVM response to UVR will weaken and be traded-off for food; a notion that may add to our understanding of the huge individual variance in DVM and avoidance behaviours in natural systems.

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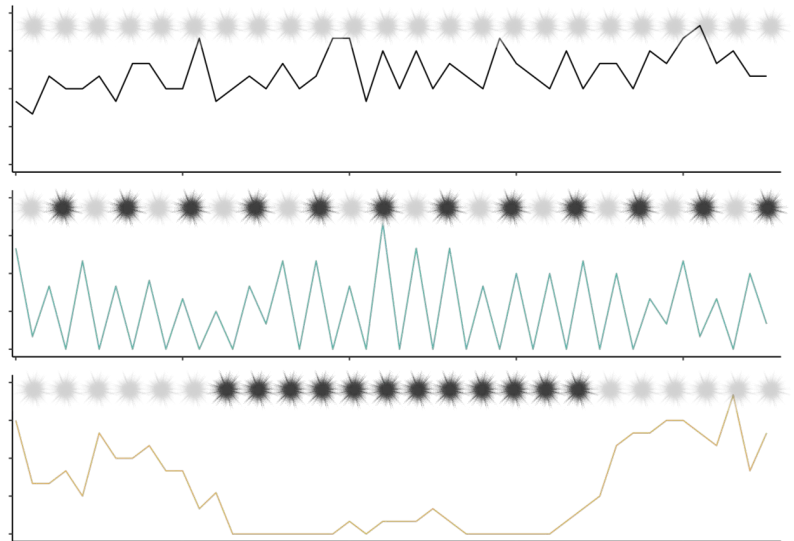
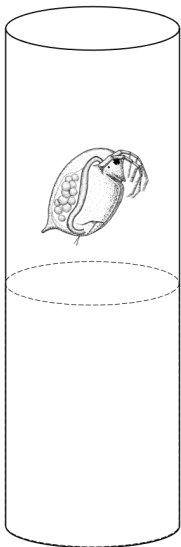
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Paper III



Research



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Population ecology

Fitness cost from fluctuating ultraviolet radiation in *Daphnia magna*

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Solar ultraviolet radiation (UVR) is an important environmental threat for organisms in aquatic systems, but its temporally variable nature makes the understanding of its effects ambiguous. The aim of our study was to assess potential fitness costs associated with fluctuating UVR in the aquatic zooplankton *Daphnia magna*. We investigated individual survival, reproduction and behaviour when exposed to different UVR treatments. Individuals exposed to fluctuating UVR, resembling natural variations in cloud cover, had the lowest fitness (measured as the number of offspring produced during their lifespan). By contrast, individuals exposed to the same, but constant UVR dose had similar fitness to control individuals (not exposed to UVR), but they showed a significant reduction in daily movement. The re-occurring threat response to the fluctuating UVR treatment thus had strong fitness costs for *D. magna*, and we found no evidence for plastic behavioural responses when continually being exposed to UVR, despite the regular, predictable exposure schedule. In a broader context, our results imply that depending on how variable a stressor is in nature, populations may respond with alternative strategies, a framework that could promote rapid population differentiation and local adaptation.

1. Introduction

In natural environments, organisms are exposed to various threats, and escaping from them generally implies a cost, in both energy and missed opportunities for feeding and reproduction. Depending on the nature, duration and predictability of the threat, different life strategies could arise [1]. In aquatic systems, solar ultraviolet radiation (UVR) is a temporally variable abiotic threat reported to have negative effects on a range of different aquatic organisms from different trophic levels, thereby structuring communities [2,3]. Increased mortality rates and reduced reproduction of several zooplankton species have been documented in response to UVR [4,5], as well as the induction of avoidance behaviours [6–8]. The zooplankton species *Daphnia magna*, in particular, has been repeatedly shown to exhibit strong negative phototaxis in response to UVR stress [8,9].

Most studies on effects of UVR have focused on effects from constant exposure as the treatment [8,10–13], despite the intensity of UVR in nature fluctuating strongly over short time scales with the position of the Sun and rapidly occurring variations in cloudiness. Several studies include these natural variations in UVR in their experimental design, but they do not explicitly address the costs of the fluctuating threat *per se* [4,14,15]. Yet, responding to these short-term fluctuations in UVR through avoidance behaviour likely implies a cost, in terms of both energy and missed opportunities for feeding and

reproduction [8]. However, in a variable but predictable environment, phenotypically plastic responses could improve individual performance [16,17]. To our knowledge, no study has addressed how long-term, continuous fluctuations in UVR, mirroring the everyday environment in natural ecosystems, affect survival, reproduction and behaviour within a single generation. Therefore, the aim of this study was to assess the costs of fluctuating exposure of UVR in *Daphnia magna*, and we hypothesized that fluctuations in UVR would reduce the number of offspring and the survival of individuals owing to the allocation of energy to threat response movements. We also hypothesized that there may be behavioural plasticity in the individual responses, possibly accounting for part of the considerable variance observed in natural ecosystems.

2. Methods

Juvenile female *Daphnia magna* (8 days old) were isolated from laboratory cultures that had been kept under constant light and temperature conditions without UVR. Three *D. magna* genotypes were used in this experiment, originally isolated from different lakes in southern Sweden. Each treatment had all three genotypes represented, with each genotype replicated at least three times per treatment (figure 1a). The individuals were isolated from the third brood of single mother per genotype. The experiment was terminated at the point when fewer than three individuals were present in all treatments (45 days).

Individual females were placed into an experimental aquarium (plexiglass tube 25 × 10 cm, height × diameter; water volume = 1635 ml), where they were kept throughout the experiment at a constant temperature of 19 ± 1°C and a 12 L:12 D photoperiod, and were fed with live *Tetrademus obliquus* (Chlorophyceae), ad libitum (details in the electronic supplementary material). To ensure standardized environments, the water in all aquaria was replaced once a week with fresh, aerated water and *T. obliquus*.

The treatments were control (C), intermittent UVR (iUV) and constant UVR (UV) (figure 1a). UVR was provided using one lamp (UVA-340 nm; Q-panel, radiation = 108.1 ± 23.5 μW cm⁻²), and daylight was provided throughout the duration of the photoperiod via a combination of cool white lamps (OSRAM L, 18W/21-840 and AURA T8 36W/830, radiation = 36.2 ± 6.2 μmol m⁻² s⁻¹). All the treatments were exposed to the same daylight intensity over the 12 h light part of the photoperiod, whereas the iUV was exposed to UVR for two periods of 15 min every hour throughout the day, mirroring fluctuating sunlight, and the UV treatment was exposed to constant UVR during 6 h a day (figure 1a), resembling a sunny day without cloud cover. The position of the aquaria within each treatment was randomized twice a week.

To determine the effects of the UVR and fluctuating exposure, *Daphnia* survival and reproduction were monitored during the entire experiment. *Daphnia* survival was checked every day and offspring were removed from the aquaria twice a week.

To assess *Daphnia* swimming behaviour, the individual position in each aquarium was registered as 'bottom' or 'surface' when the animal was below or above a line drawn at the middle of the aquarium (figure 1a). The recordings were initiated just before the UV radiation was turned on in the iUV treatment, followed by a recording about 30 s after the UV was turned on. The recordings in all treatments followed this schedule, summing up to 46 behavioural recordings during 11 h in each treatment, on four recording occasions during the experimental period.

(a) Data analysis

All analyses were performed using R v. 3.5.1 [18], and figures were drawn using the package 'tidyverse' [19]. *Daphnia* survival was analysed as a dependent variable, registered as the day of death for each individual. Survival analysis was performed using the package 'survival' [20], and Cox proportional hazard regression model, using a survival object (day of death and survival status) as the dependent variable, and treatment and genotype were used as explanatory variables.

The reproductive success of each *Daphnia* was assessed as the total number of neonates produced per female until the end of the experiment (day 45). A generalized linear model (GLM) with Poisson error distribution was used to evaluate the effects of treatments on total *Daphnia* reproduction, including also time and genotype as explanatory variables.

For the behavioural analysis, the dependent variable was the total number of changes in position performed per individual female at each sampling date, and it was analysed using a generalized linear mixed model (GLMM) with Poisson error distribution, using the package 'lme4' [21]. Date, treatment and genotype were the explanatory variables, and the individual *Daphnia* was used as random effect. All R packages used during the analysis are detailed in electronic supplementary material, table 1.

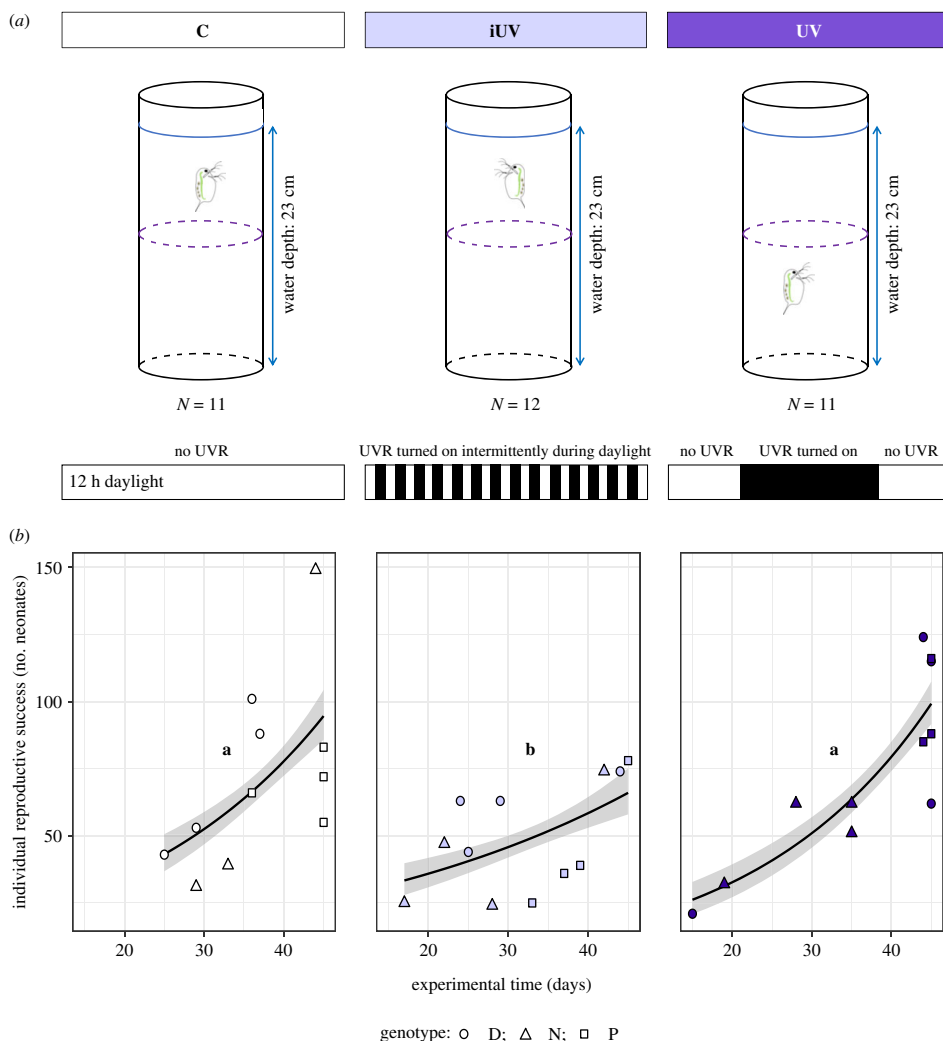
3. Results

Considering *Daphnia* survival, there were no significant differences among treatments (table 1 and electronic supplementary material, figure S1). However, individuals exposed to the fluctuating UVR treatment (iUV) showed the lowest reproductive success, measured as the total amount of neonates produced during the experiment (table 1, Tukey's test, $p < 0.05$; figure 1b). On the other hand, the UV treatment had a similar reproductive output compared with unexposed controls (Tukey's test, $p = 0.533$). The fluctuating UVR exposure reduced *Daphnia* reproductive output overall. *Daphnia* genotype was a significant variable in both survival and reproduction models (table 1).

The individuals exposed to the UV treatment (6 h of continuous dose of UVR) performed the lowest number of changes in position (table 1, Tukey's test, $p < 0.001$; figure 2) and were more often in the lower section of the aquarium compared with the iUV treatment group (electronic supplementary material, figure S2), whereas there was no difference in the number of changes in position in the iUV treatment compared with the controls (Tukey's test, $p = 0.761$; figure 2 and electronic supplementary material, figure S2). Similar to the life-history models, *Daphnia* genotype was a significant variable in the model (table 1). When analysing differences in behaviour throughout the experiment, date as an explanatory variable was not significant, i.e. we found no evidence for behavioural plasticity.

4. Discussion

Despite the importance of solar UVR in aquatic ecosystems [5,22], the consequences of natural fluctuations in UVR are still unclear. Threat responses to UVR have been repeatedly demonstrated among small invertebrates, such as *Daphnia* [6,15]. As organisms need to allocate their limited energy to body maintenance/growth, reproduction and movement, any energy diverted to repeated threat responses will not



be available for the aforementioned activities [8]. We provide here the first evidence that the variability associated with how an environmental stressor is delivered causes aquatic invertebrates to adopt alternative strategies, leading to population-level consequences.

It is well established that both UV-A and UV-B solar radiation have adverse effects on zooplankton [23]. While UV-B radiation has the potential to damage most biological macromolecules (including DNA [24]), UV-A radiation generates several by-products that cause oxidative stress to numerous

cellular components [25]. Owing to the predominant form of UVR in this study being UV-A, it is plausible to assume that the constant production of damaging chemical by-products required equally constant repairing at the molecular level. In zooplankton, this could be achieved through the utilization of either the energetically costly nucleotide excision repair process, or the less costly photo-enzymatic repair pathway, the latter being specifically induced by UV-A radiation [26]. It has been demonstrated that the induction of these systems increases the survival of individuals [27], and this could

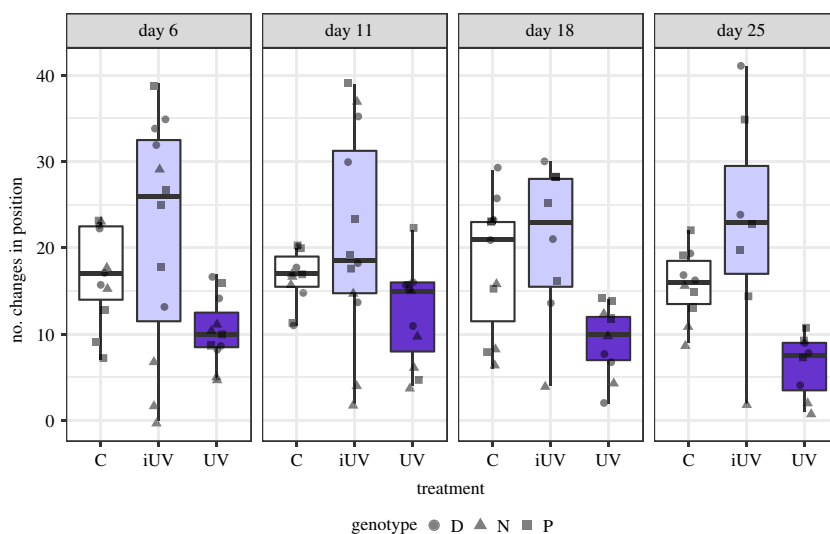


Figure 2. Number of changes in position between treatments for each behaviour recording day. The grey symbols represent the data from each individual *Daphnia* and different-shaped dots indicate different genotypes. The boxplot shows the median for each group as a black horizontal line, the first and third quartile with the box, and the minimum and maximum with the vertical lines.

Table 1. Results of Cox proportional hazard model, GLM and GLMM for survival, reproduction and behaviour, respectively. LR χ^2 tests (χ^2), degrees of freedom (d.f.) and *p*-value for each explanatory variable and interactions are shown.

dependent variable	explanatory variable	χ^2	d.f.	<i>p</i> -value
survival	treatment	3.216	2	0.200
	genotype	14.321	2	<0.001
reproduction	time	217.566	1	<0.001
	treatment	15.263	2	<0.001
	genotype	46.909	2	<0.001
	time \times treatment	13.594	2	0.011
behaviour	date	1.420	1	0.233
	treatment	30.314	2	<0.001
	genotype	23.735	2	<0.001
	date \times treatment	3.981	2	0.137

explain the absence of differences when considering survival between treatments.

The cost of the repair process could instead be covered by the redistribution of energy from other life-history traits, such as reproduction. Our results showed that individuals exposed to fluctuating UVR reduced the number of offspring produced in comparison with both non-exposed individuals and, more interestingly, those that were constantly exposed to UVR. This indicates that the dose of UVR did not determine the reproductive success; instead, it was the temporal variability of the stressor driving the different responses. As environmental variability can influence population growth and fitness in many interrelated ways [28], the fluctuating

environment in our experimental set-up could have been difficult for the organisms to predict and respond to accordingly and indeed affected the individual fitness. Our results suggest that despite the presence of the stressor in both UVR-exposure treatments, the constant environment could represent a more benign environment. The predictability of the stressor may allow behavioural adaptations to offset fitness costs.

Behavioural responses are well documented in zooplankton exposed to UVR [5–7]. In contrast to the reproductive output, the behaviour of individuals exposed to fluctuating UVR closely resembled the behaviour of the non-UVR-exposed *Daphnia*. Constantly exposed individuals, however, showed a dramatic reduction in daily movement. We considered the possibility that over time *Daphnia* can plastically adapt behaviourally, but we found no evidence supporting plastic behavioural responses in this experiment. *Daphnia* have long been established to be negatively phototactic, with extreme avoidance of UVR [9]. UVR has, in fact, been proposed as one of the key drivers in the iconic diel vertical migration pattern that *Daphnia* and many other zooplankton perform [6]. Although the costs of diel vertical migration have been assumed negligible [29,30], there has been some controversy over the energetics of such movements [8]. Our results clarify this by showing that the movements to repeatedly avoid UVR may, indeed, increase energy demands. This is based on the observation that the iUV-treated group was the only one that had a reduction in reproductive output. On the other hand, the group exposed to constant UV showed reduced movement, staying in deeper water during exposure, with no identifiable consequence to reproduction.

We recognize that in more natural settings, the heterogeneity of the environment would allow behaviour to play a larger part in determining the optimal strategy for maximizing fitness. For example, diel vertical migration,

for which many zooplankton species are renowned, drastically alters exposure to UVR. Hence, extrapolating behavioural results from controlled experiments into natural environments should be done with caution. Despite these precautions, we show here that *Daphnia* have the potential to adopt alternative strategies for dealing with either constant exposure or repeatedly fluctuating UVR, and the response to the more variable environment represents a higher reproductive cost. It has been demonstrated that other threats, such as predation, can cause rapid, local adaptations [31,32]; in a broader context, the significant effect of genotype in our study implies that, depending on how variable a stressor is in nature, the population responses can be different, generating a framework that likely can promote rapid population differentiation and local adaptation.

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Supplementary Information

Fitness cost from fluctuating ultraviolet radiation in
Daphnia magna

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Feeding of *Daphnia*

The water in all aquaria was replaced once a week with fresh, aerated water. Algal food from a culture of *Tetrademus obliquus* was added three times a week throughout the experiment. The algae culture concentration was approximately 1465 µg Chlorophyll-a/L. At each weekly water replacement, 30 mL from this culture was mixed with fresh water in each aquarium, leading to an algae concentration of 27.4 µg Chl-a/L per *Daphnia* individual. At the two other feeding occasions, 5 mL of the same culture was added to each aquarium, ensuring that each individual was fed *ad libitum*.

Table S1. List of R packages that were used during the data analysis and their reference.

	R package or library name	Reference
1	tidyverse	Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H (2019). "Welcome to the tidyverse." <i>Journal of Open Source Software</i> , 4(43), 1686. doi: 10.21105/joss.01686.
2	ggplot2	Wickham H (2016). <i>ggplot2: Elegant Graphics for Data Analysis</i> . Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org .
3	lme4	Bates D, Mächler M, Bolker B, Walker S (2015). "Fitting Linear Mixed-Effects Models Using lme4." <i>Journal of Statistical Software</i> , 67(1), 1–48. doi: 10.18637/jss.v067.i01
4	car	Fox J, Weisberg S (2019). <i>An R Companion to Applied Regression</i> , Third edition. Sage, Thousand Oaks CA. https://socialsciences.mcmaster.ca/jfox/Books/Companion/ .
5	DHARMA	Florian Hartig (2019). DHARMA: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.2.6. https://CRAN.R-project.org/package=DHARMA
6	ggfortify	Yuan Tang, Masaaki Horikoshi, and Wenxuan Li. "ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages." <i>The R Journal</i> 8.2 (2016): 478–489. Masaaki Horikoshi and Yuan Tang (2016). <i>ggfortify: Data Visualization Tools for Statistical Analysis Results</i> . https://CRAN.R-project.org/package=ggfortify
7	MuMIn	Kamil Bartoń (2019). MuMIn: Multi-Model Inference. R package version 1.43.6. https://CRAN.R-project.org/package=MuMIn
8	survival	Therneau T (2015). <i>_A Package for Survival Analysis in S_</i> . version 2.38, <URL: https://CRAN.R-project.org/package=survival >. Terry M. Therneau, Patricia M. Grambsch (2000). <i>Modeling Survival Data: Extending the Cox Model</i> . Springer, New York. ISBN 0-387-98784-3
9	lmerTest	Kuznetsova A, Brockhoff PB, Christensen RHB (2017). "lmerTest Package: Tests in Linear Mixed Effects Models." <i>Journal of Statistical Software</i> , 82(13), 1-26. doi: 10.18637/jss.v082.i13 (URL: https://doi.org/10.18637/jss.v082.i13)
10	lmtest	Achim Zeileis, Torsten Hothorn (2002). <i>Diagnostic Checking in Regression Relationships</i> . <i>R News</i> 2(3), 7-10. URL https://CRAN.R-project.org/doc/Rnews/
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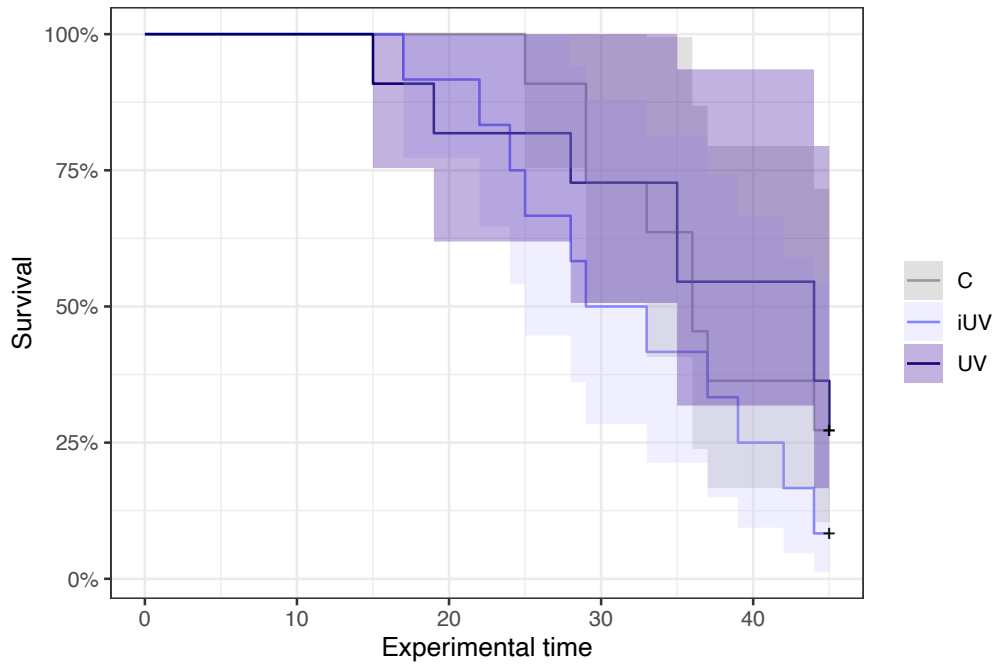


Figure S1. Kaplan-Meier survival plot showing the survival through the experimental time (in days) for the three treatments: control (non-UVR-exposed individuals, in grey), iUV (individuals exposed to fluctuating UVR, in lilac) and UV (individuals exposed to constant UVR, in violet).

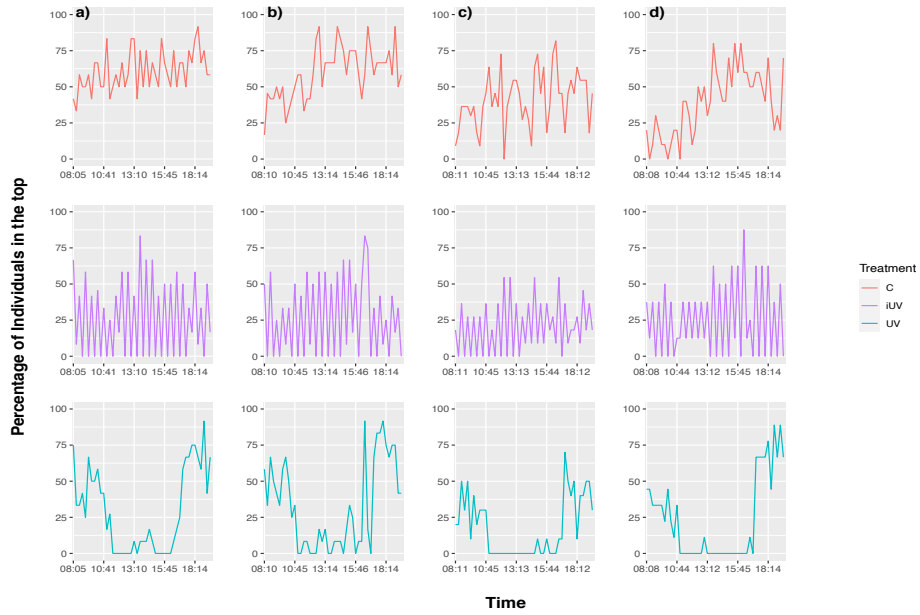
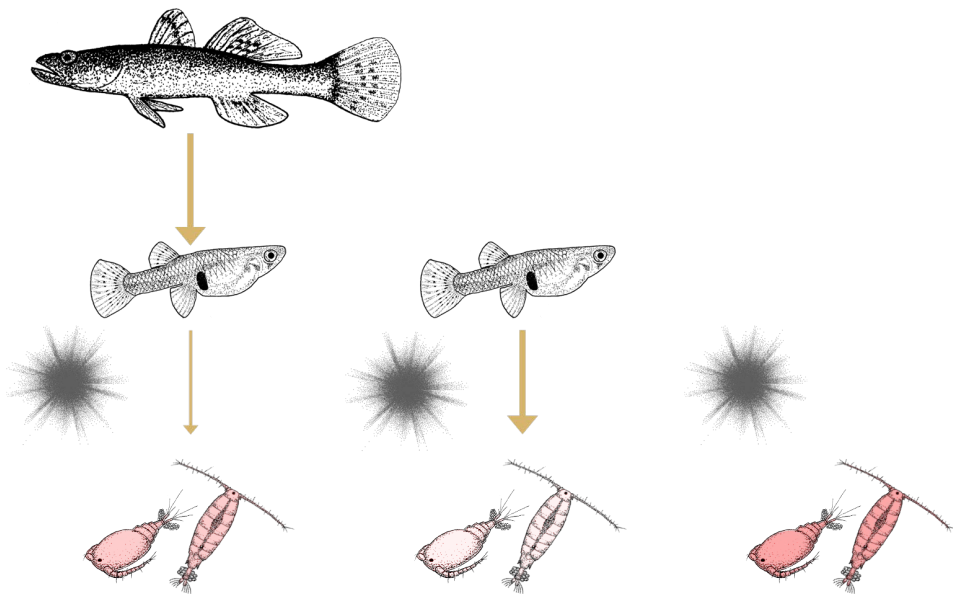


Figure S2. Percentage of individuals that were in the top of the aquaria during day-time for each treatment (control in red, iUV in purple and UV in skyblue) and for each behavioural sampling date: column a) experimental day 6, column b) experimental day 11, column c) experimental day 18, column d) experimental day 25.



Research



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Low-latitude zooplankton pigmentation plasticity in response to multiple threats

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Crustacean copepods in high-latitude lakes frequently alter their pigmentation facultatively to defend themselves against prevailing threats, such as solar ultraviolet radiation (UVR) and visually oriented predators. Strong seasonality in those environments promotes phenotypic plasticity. To date, no one has investigated whether low-latitude copepods, experiencing continuous stress from UVR and predation threats, exhibit similar inducible defences. We here investigated the pigmentation levels of Bahamian 'blue hole' copepods, addressing this deficit. Examining several populations varying in predation risk, we found the lowest levels of pigmentation in the population experiencing the highest predation pressure. In a laboratory experiment, we found that, in contrast with our predictions, copepods from these relatively constant environments did show some changes in pigmentation subsequent to the removal of UVR; however, exposure to water from different predation regimes

induced minor and idiosyncratic pigmentation change. Our findings suggest that low-latitude zooplankton in inland environments may exhibit reduced, but non-zero, levels of phenotypic plasticity compared with their high-latitude counterparts.

1. Introduction

All organisms are perpetually exposed to information conveying both threats and opportunities [1,2], and each individual organism must act upon this information to maximize its opportunities while simultaneously minimizing the risk from threats. In prey organisms, a common strategy to reduce risk in the face of increasing threat from predators is to induce defence traits, such as specific behaviours, morphologies and chemicals that reduce an individual's vulnerability to predation [3,4]. Inducible defences are favoured when there is temporal variability in predation pressure and when prey have reliable means of evaluating predation risk [3], whereas temporally homogeneous environments should select for canalized phenotypes [5], i.e. prey defence traits that are constitutive and locally adapted to the prevailing predation regime. Furthermore, inducible defence traits should incur costs to the individual's fitness which prevents them from becoming constitutive defences, which are expressed even in the absence of the threat [6]. Despite their ephemeral nature, plastic defensive traits have profound effects for both direct and indirect interactions with other organisms, which make them both ecologically and evolutionarily influential [7].

In aquatic systems, zooplankton comprise a long-standing and valuable model for investigating inducible defences [8–12]. They are amenable to laboratory experimentation and occupy an integral position in aquatic food webs, filling the role of primary consumers as well as being an indispensable prey source for most larval and many adult fish [13]. Zooplankton frequently induce modifications in morphology, physiology and behaviour to gain protection from predators [9,12,14,15]. For example, rotifers such as *Keratella* spp. can induce spine elongation when exposed to the predaceous rotifer *Asplanchna* sp. [16] or decrease spine length when threatened with fish predators [17]. Cladocerans, particularly in the genus *Daphnia*, boast a plethora of inducible defences: they form helmets, invest in longer tail spines, induce diapause and alter swimming behaviours, all in response to predator cues [6,9,18,19]. These predator cues, or kairomones, are detected through chemoreception which informs the zooplankton of general rather than acute predation risks [20].

Threats do not only arise from the risk of predation, however. Solar ultraviolet radiation (UVR) is another well-documented stressor for zooplankton, eliciting multiple forms of inducible protection [8,14]. Copepods, a common and important group of zooplankton, have demonstrated inducible defences in response to UVR exposure [2,21]. A common strategy is to accumulate photoprotective compounds such as melanin, mycosporine-like amino acids or carotenoids [22]. In environments where UVR is a substantial threat, such as clear lakes which allow UVR to penetrate deeper [23], copepods have been shown to contain large quantities of carotenoids, which confer protection from UVR through the neutralization of free radicals [24,25]. In their free form or as bound lipids, carotenoids appear red or yellow [22]. This pigmentation can make individuals more conspicuous targets for visually hunting predators such as fish [26]. Therefore, it should be predicted that copepods in environments with fish predators will have lower levels of pigments than nearby populations without visually hunting predators. Numerous studies—primarily at high latitudes or in high-elevation lakes—have demonstrated that this trade-off exists and that copepods can rapidly adjust pigmentation levels in response to changes in predation cues or UVR [2,14,21,26,27]. Due to seasonal changes in these environments, there is a substantial variation in both UVR and predation levels across the year, and this variation is a key feature for the promotion of phenotypic plasticity and inducible defences [3,28].

Multiple ecological and environmental differences between temperate and low-latitude systems can influence the plasticity of pigmentation. Fish reproductive periods, for example, are more constrained towards the poles as fish typically reproduce once annually, whereas fish in the subtropics are fractional spawners resulting in a less variable predation regime analogous to the climatic variability hypothesis [29,30]. Similarly, UVR in high-latitude environments is particularly stressful during summer, but the threat is completely absent during winter months; in the subtropics, however, the threat of UVR is still variable but never absent (figure 1). To our knowledge, no studies yet have investigated the plasticity of pigmentation at lower latitudes.

Bahamian 'blue holes', which are water-filled vertical caves with a freshwater layer floating atop marine ground water [31], represent a unique opportunity to investigate zooplankton pigmentation and the role of phenotypic plasticity in the subtropics. They represent isolated, temporally stable environments that are very simple with regard to the trophic webs as they only contain a small number of species [31].

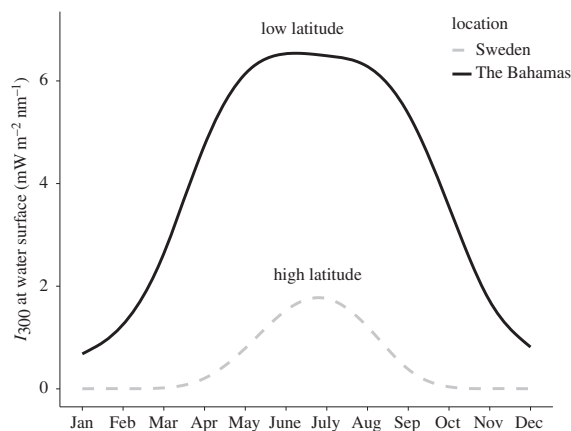


Figure 1. Estimation of surface irradiance of UV-B (300 nm) at a high-latitude location (Lund, Sweden; 55.71°N, 13.20°E) and a low-latitude location near the focal study sites (Nassau, The Bahamas; 25.05°N, 77.40°W) across the year during 2017. Data computed from the 'FASTRTR' model V2.3 (<https://fastrtr.nilu.no>) and computed as the first day of each month at noon with cloudless conditions.

Importantly, as these blue holes were formed thousands of years ago, the zooplankton in these systems have evolved with different apex predators: some blue holes have both piscivorous and zooplanktivorous fish, others have only zooplanktivorous fish and a few lack vertebrate predators altogether [31]. This range of stable predator regimes, coupled with the intense while still variable, year-round UVR, allows us to make explicit predictions regarding the level of pigmentation expected in copepods inhabiting environments with conflicting threats.

The objective of the present study was to identify whether low-latitude copepods show similar phenotypic patterns to those from high latitudes when exposed to UVR and predation pressure. We hypothesized that the high and more constant UVR exposure across the subtropics requires year-round protection in all blue holes and, hence, by comparing blue holes that differ in fish assemblage, we could test the hypothesis that copepods from environments with greater threats of predation from visually hunting predators would have lower levels of photoprotective pigmentation. Specifically, we predicted that zooplankton in environments with no predators should have the highest level of pigmentation, those with only zooplanktivorous fish would have the lowest, and due to the reduced yet not absent predation pressure, those with both zooplanktivorous and piscivorous fish would have an intermediate level. Furthermore, in a laboratory experiment, we tested the prediction that low-latitude copepods, unlike copepods from higher latitudes, will not exhibit phenotypic plasticity in pigmentation due to the low temporal variation in predation intensity and the continual presence of UVR over the year, with any phenotypic differences between populations instead representing constitutive defences.

2. Material and methods

2.1. Field sampling

Copepods were collected from three blue holes on Andros Island, The Bahamas, during March 2018. Blue holes, although sharing most characteristics, do vary in many features such as surface area, freshwater depth and turbidity. Therefore, we selected these blue holes *a priori* primarily based upon the presence/absence of zooplanktivorous and piscivorous fish [31] while also considering the geographical proximity to one another and the similarity of the UVR threat among blue holes (see electronic supplementary material). Turtle Blue Hole (24°46'21.72"N, 77°51'5.472"W) has no fish, hereafter 'no-predation'; Cousteau's Blue Hole (24°46'33.6"N, 77°54'57.6"W) harbours a population of a piscivorous fish species (bigmouth sleeper, *Gobiomorus dormitor*) and a relatively low density of a zooplanktivorous fish (Bahamas mosquitofish, *Gambusia hubbsi*), hereafter referred to as 'low-predation'; and Rainbow Blue Hole (24°47'6"N, 77°51'36"W) has no piscivorous fish and a high density of *G. hubbsi*, hereafter 'high-predation'. We estimated the daytime predation risk of small aquatic prey in each blue hole by quantifying the average number of bites towards possible prey by fish per minute per cubic metre

(see electronic supplementary material). These estimates confirmed our expectation that Rainbow Blue Hole experienced the highest predation risk, followed by Cousteau's Blue Hole, and then Turtle Blue Hole which had no fish predators (electronic supplementary material, table S1). To collect the copepods, we sampled the entire freshwater layer of each blue hole by lowering a 100 μm net, with a mouth diameter of 30 cm, and gently retrieving it. We collected a sample of concentrated zooplankton for immediate pigmentation analysis and another sample for a laboratory experiment (see below). We found that no- and high-predation systems contained only calanoid copepods and the low-predation system contained only cyclopoid copepods. As we were interested in general patterns and both types of copepods exhibit plasticity in pigmentation at higher latitudes [2], we included both in our laboratory experiment.

2.2. Experimental design

To test whether pigmentation in copepods at lower latitudes is a phenotypically plastic trait that responds to changes in UVR and predation risk, we performed a laboratory experiment. To assess the influence of predation risk, we employed a 3×3 factorial experimental design (3 populations \times 3 treatments) with five replicates each. Population represented the initial predation regime (no-, low- and high-predation risk) of the population and treatments represented differences in perceived predation risk (chemical predator cues). We devised our treatments using water from the three blue holes filtered through a 50 μm mesh to remove other large zooplankton but keep both phytoplankton and the chemical cues from any potential predators. We collected experimental animals using the methodology above, and all were collected on the same day and brought to the laboratory where there was a 12 : 12 light : dark photoperiod with no exposure to UVR. This absence of UVR allowed us to explicitly test whether the pigmentation of these copepods is plastic in response to UVR as well as perceived predation risk. For each regime (no-, low- and high-predation risk, respectively), we filled fifteen 300 ml containers with water collected in the field and again filtered through a 50 μm mesh. We then took the zooplankton samples from the field and divided each population into each container: five replicates of the no-predation risk system water, five replicates of the low-predation risk system water and five replicates of the high-predation risk system water (45 total containers, 5 replicates per population \times treatment combination). This was achieved by gently mixing the field samples before taking a 200 ml subsample, thereby coarsely standardizing the number of animals, filtering the water away using the 50 μm mesh and carefully introducing the zooplankton on the mesh to the treatment container.

The laboratory experiment was run for 10 days, as it has been shown that carotenoid content can adjust to changed risk levels after only 4 days [21]. To maximize the predator cues, while simultaneously limiting mechanical disturbance to the copepods, 100 ml of water from each container was exchanged every other day. This was achieved by slowly filtering through a 50 μm mesh (to ensure that experimental zooplankton were not lost) and replaced with 100 ml of filtered treatment water collected that same day from the respective blue hole. This method also supplemented each container with fresh phytoplankton and micro-zooplankton for both the herbivorous and omnivorous copepods. At the end of the experimental period (10 days), each container was randomly selected, gently mixed and the water filtered through a 50 μm mesh to collect the copepods. Only live adult copepods were photographed for the quantification of pigmentation using the methodology described below.

2.3. Quantification of pigmentation

To quantify pigmentation, live copepods were gently transferred to an individual drop of glycerol on a glass slide using forceps. To avoid any damage to the cephalosome (our area of interest, as to minimize the risk of measuring the green gut material), animals were manipulated by their antennae. We then took a digital photo of each copepod at 200 \times magnification using a Dino-Lite Edge X 200 \times (USB3) microscope (AnMo Electronics Corporation, Taiwan) and the associated DinoXcope software. All copepods were manipulated into the same position when taking photos, and light conditions were standardized by taking the photos in a darkened room with only the focal light on the subject. To assess pigmentation levels, the photos were subjected to a profile conversion in Adobe Photoshop CC 2017, following Brüsün *et al.* [15]. Here, the colour profile is changed from RGB to Lab Colour, which is based upon the standardized, device independent colour space, CIE (Commission International de l'Eclairage) $L^*a^*b^*$. Still in Photoshop, the 'Quick Selection tool' was used to select the cephalosome, and the mean 'redness' (a^*) and 'yellowness' (b^*) in that given selection were obtained from the in-built histograms. The values for those colour channels range from 0, which appears as true green (a^*) or true blue (b^*) to the human eye, to 255, which appears true red (a^*) or true yellow (b^*). Together, these two attributes of colour have successfully been applied as proxies for carotenoid-based coloration [32,33].

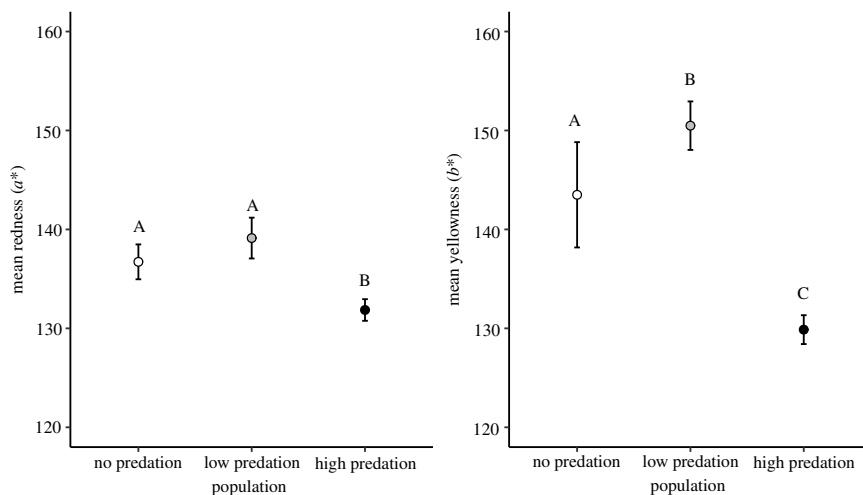


Figure 2. Mean copepod pigmentation levels (a^* and b^*) \pm 2 s.e. among three Bahamian blue holes with different food chain lengths and, therefore, predation risk ranging from no fish (no-predation risk, white bars), both zooplanktivorous and piscivorous fish (low-predation risk, grey bars) and only zooplanktivorous fish (high-predation risk, black bars) ($n = 6$ per lake). Horizontal lines represent the mean with the box denoting the 25th and 75th percentiles and the whiskers representing the 5th and 95th percentiles. Letters denote significant difference between populations ($p < 0.05$).

2.4. Statistical analysis

All analyses were performed using R v. 3.4.3 [34]. To test for differences among the blue hole populations, we conducted two separate ANOVAs using redness and yellowness values, respectively, as dependent variables and employed the *post hoc* Tukey's test to examine differences between populations if the main effect was statistically significant ($p < 0.05$). To examine effects of population and water source (i.e. predation cue) in the laboratory experiment, we calculated the change (Δ) in pigmentation by subtracting the colour values of the zooplankton exposed to our treatments at the end of the experiment from the average pre-experiment value of the population from which they originated. We then performed separate linear mixed models using Δa^* and Δb^* values as dependent variables with the package 'lme4' [35]. Treatment, population and their interaction served as fixed effects, while replicate ID was entered as a random effect as five individuals were taken from each container, yielding a sample size of 25 for each population \times treatment combination.

3. Results

3.1. Population differentiation

Field-collected copepods exhibited clear differences in pigmentation between populations (a^* : $F_{2,15} = 19.24$, $p < 0.0001$; b^* : $F_{2,15} = 36.17$, $p < 0.001$; figure 2). In accordance with our predictions, environments with only zooplanktivorous fish (high predation) had the lowest levels of carotenoid pigmentation. This high-predation regime exhibited lower levels of both redness and yellowness compared with those from the fishless environment (Tukey's tests: $p = 0.003$ and $p < 0.001$, respectively) or compared to those from the low-predation system (Tukey's tests: both $p < 0.001$). Unexpectedly, the cyclopoid copepods from the low-predation system had similarly high levels of redness as the copepods from the no-predation system (Tukey's test, $p = 0.143$) and even greater levels of yellowness (Tukey's test: $p = 0.032$).

3.2. Laboratory experiment

As UVR is continually present over the year at low latitudes, we expected little-to-no effects of UVR removal across populations in the common garden environment. However, contrary to our expectations, we found

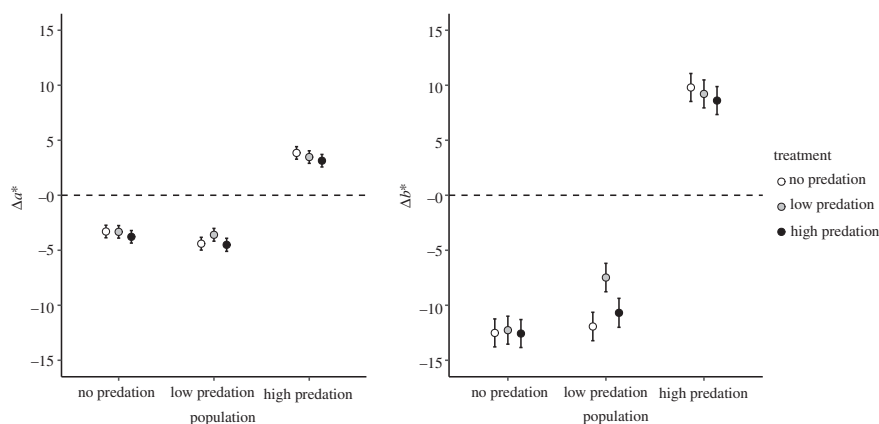


Figure 3. Change in mean pigmentation levels (Δa^* = redness and Δb^* = yellowness) of copepods ($n = 25$ per treatment \times population) due to the removal of UVR and exposure to predatory cues in the laboratory experiment compared with the mean pigmentation of samples taken directly from the respective blue hole (hatched line). The values depicted here represent the least square means and the ± 2 standard error taken from the independent mixed models, accounting for the effect of the replicates. Increases in pigmentation are denoted by positive values and losses are denoted by negative values. All changed significantly from the baseline data ($p < 0.05$).

Table 1. Results from linear mixed-model analyses of changes in pigmentation, separately examined using redness (a^*) and yellowness (b^*) colour variables, during the laboratory experiment. Significant results are italicized.

factor	colour channel	<i>F</i> statistic	d.f.	<i>p</i> -value
treatment	<i>a</i> *	3.14	2,35.3	0.056
	<i>b</i> *	4.60	2,32.5	0.017
population	<i>a</i> *	654.21	2,35.3	<0.001
	<i>b</i> *	1040.40	2,32.5	<0.001
treatment \times population	<i>a</i> *	1.12	4,35.3	0.362
	<i>b</i> *	4.57	4,32.5	0.005

that the removal of the UVR threat resulted in clear changes in the pigmentation levels, albeit in different directions in the different populations (table 1 and figure 3; electronic supplementary material, figure S1). Regardless of the predation risk treatment, the no- and low-predation risk populations lost pigmentation (in both redness and yellowness) in the absence of UVR, whereas the high-predation risk population increased pigmentation. Second, we expected that due to the lack of temporal variation in predation pressure, we would not find plasticity in response to differing predation risk chemical cues. Contrary to our expectations, the introduction of water from alternative blue holes with contrasting predation regimes had minor effects upon copepod pigmentation (table 1 and figure 3). The effects of the treatment on Δa^* were weak and marginally non-significant, although the treatment effects on Δb^* were more pronounced (table 1). Lastly, we investigated whether there was an interaction between population and the treatment effects. We found no significant evidence for an interaction between treatment and population in Δa^* but we did for Δb^* (table 1). This result appears to be driven by the zooplankton in the low-predation system, which reduced their yellowness more when exposed to foreign water compared to water from their own blue hole (figure 3).

4. Discussion

There is a pronounced bias in plankton plasticity research towards high latitudes and high elevations where environmental conditions are highly seasonal and variable [11,21,24,26]. As such, it is unknown

whether zooplankton from low latitudes (i.e. subtropics and tropics) exhibit plasticity in phenotypic responses to divergent threats such as predation and UVR, as found in other systems. We postulated that copepods in the subtropics would follow similar among-population phenotypic patterns in pigmentation to those found at higher latitudes with respect to the prevailing threat, yet they would represent constitutive defences due to invariable predation risk and consistently present solar UVR, rather than the plastic responses so characteristic of copepods in higher latitudes [2]. Specifically, we hypothesized that copepods from environments with no visually hunting predators would have higher levels of photoprotective pigmentation than those exposed to fish. We also hypothesized that when removed from UVR and exposed to water from different predation regimes, they would not adaptively alter pigmentation according to the new threat regime.

Using the natural Bahamian blue hole system, we have been able to demonstrate that low-latitude freshwater copepods do in fact display similar among-population pigmentation patterns compared to their high-elevation and high-latitude counterparts [2,14]. That is to say, as predicted, calanoid copepods in an environment with zooplanktivorous fish (high predation) had less redness and less yellowness than those from the environment without visually hunting predators (no-predation). This finding is well aligned with the available literature concerned with the trade-offs in pigmentation protection and the presence of visually hunting predators, which suggests that the accumulation of carotenoid compounds appears to be restricted to environments from which fish predators are absent [2,21]. Despite the difference in species composition, we confirmed our prediction that the environment with both piscivorous and zooplanktivorous fish (low-predation) would have a higher level of pigmentation than the high-predation system due to reduced predation intensity through the predator effects on *Gambusia hubbsi* by *Gobiomorus dormitor*. However, our prediction was not met regarding the difference between no- and low-predation risk systems, which may be partly due to taxonomic differences of the copepod assemblage among blue holes. The low-predation risk system was dominated by cyclopoid copepods, whereas the other systems were dominated by calanoid copepods. Even if it is known that both cyclopoid and calanoid copepods increase pigmentation when exposed to high UVR levels, it is possible that taxonomic differences may affect pigmentation levels among these systems as it is known that even copepods from within the same family show differences in the ability to sequester pigmentation [36]. Therefore, despite the fact that cyclopoid and calanoid copepods have been shown to exhibit similar levels and seasonal variations in pigmentation at higher latitudes [2], we cannot state whether the level of predation or the independent evolution of calanoids and cyclopoids have led to the different levels of pigmentation observed here. Increasing the number of investigated lakes with different trophic levels from one per treatment would provide far clearer information on the relative importance of predation in pigmentation.

Having determined that there were phenotypic differences between differing species and predation regimes, the next step was to investigate whether the populations exhibited pigmentation plasticity in response to reduced UVR or changes in perceived predation risk. At higher latitudes, changes in UVR and predation pressure have been repeatedly demonstrated to induce pigmentation changes in copepods [2,21,22,24,26]. Copepods have also been shown to behaviourally respond to UVR and can therefore clearly sense the presence of UV wavelengths [37]. In these higher latitude environments, there are periods when UVR is absent and it is then beneficial to have low levels of carotenoids that are costly to maintain. As such, we posited that pigmentation would not be a plastic trait in the lower latitudes due to the constant presence of UVR year-round even though there is variability within the year (figure 1). Despite our predictions, we found that pigmentation is a plastic trait in the low-latitude blue hole systems.

The removal of UVR caused copepods from all populations to change their pigmentation. Copepods from the no- and low-predation systems reduced pigmentation, similar to other UVR removal experiments [27], whereas those from the high-predation system increased pigmentation levels (figure 3). We assume that copepods in high-predation systems are exposed to a high and constant threat of predation, and an adaptive behaviour in this environment towards both UVR and predation could be to avoid the surface waters during the day via diel migration, as opposed to the reduction in damage through carotenoid usage. Copepods are capable of detecting depth through the combination of hydrographic and optical features [38]. Our experiment mimicked surface waters and prevented the diel vertical migration behaviour that is possible in the blue hole system; therefore, the copepods may have increased pigmentation to protect against the surface UVR regime they have evolved to expect irrespective of predation, leading to the observed pattern. Furthermore, in the absence of hydrodynamic disturbances caused by fish predators, copepods from the high-predation population may have increased pigmentation in response to a perceived reduction in predation risk irrespective of the water-cue treatment [39].

In contrast with UVR conditions, changes in exposure to non-familiar predator cues had either no or only a minor effect on copepod pigmentation. The change in redness in copepods treated with water from the other populations was small (marginally non-significant treatment effects and no interaction effect), corresponding to our initial prediction that there would be no plasticity. The water-cue treatment had, however, a significant effect on yellowness, yet this appears to be driven by the low-predation population dominated by cyclopoid copepods. Water cues only altered yellowness in the low-predation system, where cyclopoid copepods exhibited reduced yellowness when exposed to water from either a no-predation or high-predation environment. This neither follows adaptive predictions nor our initial hypothesis. Carotenoids in copepods must be sequestered from the food source and can appear as either red or yellow. Consequently, the low-predation risk system may have had a phytoplankton composition with a higher proportion of 'yellow' carotenoid species, such as lutein that is found in Prasinophyceae and Chlorophyceae [40], than in the other systems. If this was true, however, it would be expected that the calanoid copepods from no- and high-predation systems would also have higher levels of Δb^* when exposed to low-predation treatment water. But, as this was not the case, it appears that both pigmentation sequestering and the plasticity of this trait in this system vary among taxonomic groups as found in other studies [15,36].

Our initial prediction was that the temporal consistency in predation pressure and the intensity of UVR of the low-latitude blue holes would lead to canalized phenotypes. However, the clear and pronounced changes in pigmentation when UVR threat was removed indicate that these copepods have a pigment defence against UVR that is phenotypically plastic, not entirely constitutive. This may be due to the annual variation in UVR also present in the subtropics (figure 1). Despite UVR being a constant threat, i.e. never zero irradiance, like the winter months in the higher latitudes, the variation present could still be sufficient to promote plasticity. Furthermore, changes in cloudiness may create variable UVR conditions at a smaller temporal scale. As for plasticity in response to predation, we found only minor and idiosyncratic responses, fitting our expectations based on the temporal consistency in predation pressure in this system. It is possible that factors that promote and maintain plasticity, other than temporal variation in predation threat, may explain the minor degree of plasticity we observed here. For example, infrequent migration of copepods between blue holes that differ in the predation regime may contribute to the evolution and maintenance of plasticity [41]. We believe that our findings add to the mounting evidence that copepod plasticity is not as highly constrained at lower latitudes as earlier thought [42]. Specifically, the pigmentation response to UVR across all copepods is less constrained than previously thought, possibly due to the underestimation of the variation in the environmental cue. Further studies should address the mechanisms maintaining plasticity in low-latitude environments.

We conclude that zooplankton from different populations have differing pigmentation based upon the prevailing threat combination. Calanoid copepod populations in Bahamas blue holes exhibited pigmentation patterns matching predictions based on predation threat, similar to patterns previously observed at higher latitudes. High-latitude zooplankton also show adaptive plasticity in pigmentation in response to predator cues, while we here found that low-latitude calanoid copepods showed little evidence of plasticity and cyclopoid copepods exhibited only minor plasticity inconsistent with adaptive hypotheses.

Ethics. To collect zooplankton, no ethical permissions were required at that time. We were also not required to complete an ethical assessment prior to conducting our research as copepods are not a protected or legislated group. All animals were euthanized as humanely as possible at the end of the experiment and destroyed to prevent secondary toxicity. Fieldwork was conducted under R.B.L.'s permission from The Bahamas government.

Data accessibility. Raw data files, images used for the data collection and the R code used to analyse that data are available online in the Dryad Data Repository at: <https://doi.org/10.5061/dryad.bd4486s> [43]. Variables used to *a priori* select focal blue holes and a further figure describing the variation in the experiment are available within the electronic supplementary material.

Authors' contributions. M.L., H.Z., Y.S., A.H. and L.-A.H. conceived and led the study. M.L., R.B.L. and P.A.N. conducted the analyses. M.L. wrote the first version with substantial contributions from R.B.L. and C.B. All authors aided in fieldwork and provided valuable guidance both during the research process and the revision of the manuscript.

Competing interests. Authors declare no competing interest and confirm that the manuscript has not been submitted elsewhere for publication.

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Supplementary Information

Low-latitude zooplankton pigmentation plasticity in response to multiple threats

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Additional Site Information

We selected blue holes geographically near one another (all within a 3.7 km² region) that all exhibit relatively similarly clear water. Based on 22 turbidity measurements taken with an Oakton T-100 turbidimeter (Vernon Hills, IL) in these blue holes from 2011 to 2018, sites range in turbidity from 0.55 NTU (Cousteau's) to 0.65 NTU (Rainbow) to 0.68 NTU (Turtle), and do not significantly differ from each other (ANOVA: $F_{2,19} = 0.54$, $p = 0.59$). Average water transparency in these blue holes is 7.3 m based on Secchi disk measurements. Thus, UVR exposure is likely very similar across all three localities.

Quantification of Predation Risk

Temporally repeatable estimates of fish densities have been measured in these blue holes using underwater visual census (Heinen et al. 2013, Martin et al. 2015). Moreover, the total number of bites taken toward possible prey per minute during daylight hours for individual adult male and female Bahamas mosquitofish have also been estimated for these blue holes (Heinen-Kay et al. 2016). Multiplying sex-specific feeding rates by sex-specific density, and assuming that potentially zooplanktivorous bigmouth sleepers (less than 10cm total length) take one bite per minute, we calculated an estimate of total daytime predation threat for small aquatic prey in each blue hole containing fish (Table S1). We recognise that, although Turtle has no fish predators, it likely has invertebrate predators which may hunt visually and therefore the true predation risk is greater than zero.

Table S1. Predation risk as quantified by observing the total number of bites toward possible prey by fish per minute per cubic meter during daylight hours.

Blue hole	Predation risk min ⁻¹ m ⁻³
Turtle	0
Cousteau's	11.59
Rainbow	35.61

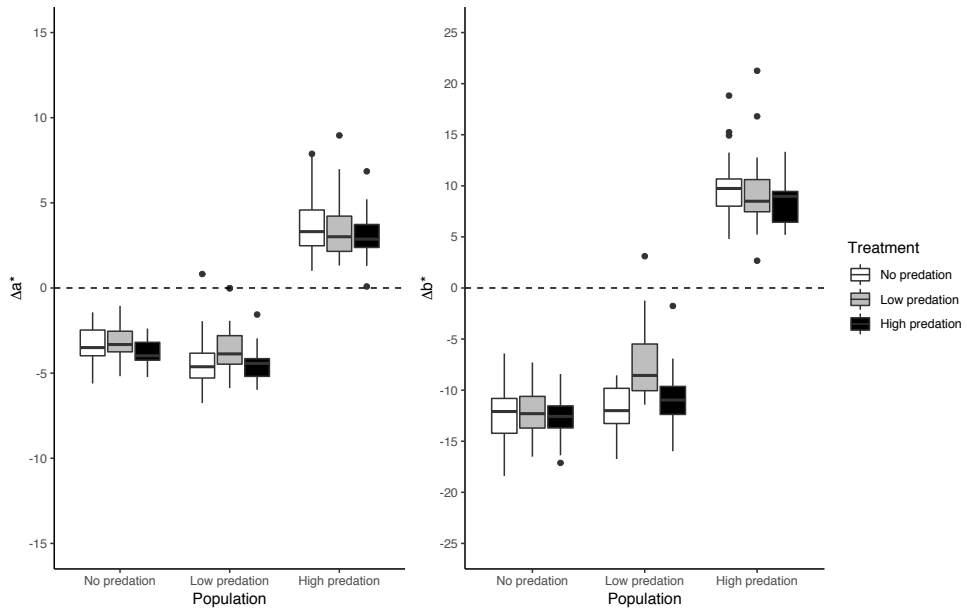
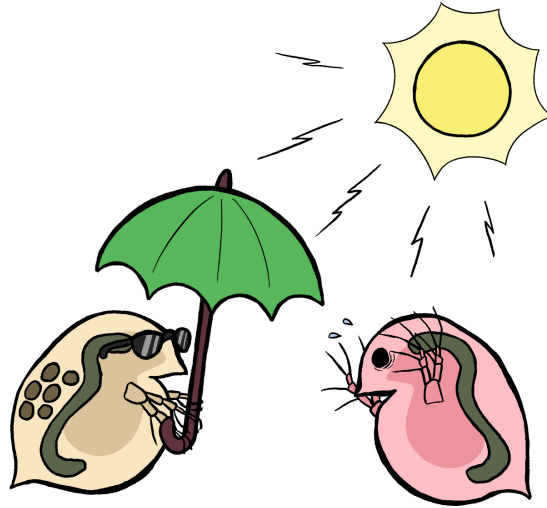


Figure S1. Raw data displaying the variation in median pigmentation levels (Δa^* = redness, Δb^* = yellowness) of copepods ($n = 25$ per treatment X population) after exposure to the treatments in the laboratory experiment compared with the mean pigmentation of samples taken directly from the respective blue hole (hatched line). Note that the axes are different between the two pigments, yet the patterns are consistent.

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List of papers

- I. Lee, M., Solano Udina, C. & Hansson, L.-A. 2021 Fear of sex: sexual conflict exposed as avoidance in a parthenogenetic invertebrate. *Behavioural Ecology and Sociobiology* 75, 115. <https://doi.org/10.1007/s00265-021-03054-9>
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- IV. Lee, M., Zhang, H., Sha, Y, Hegg, A., Ekelund Ugge, G., Vinterstare, J., Škerlep, M., Pärssinen, V., Herzog, S.D., Björnerås, C., Gollnisch, R., Johansson, E., Hu, N., Nilsson, P.A., Hulthén, K., Rengefors, K., Langerhans, R. B., Brönmark, C., Hansson, L.-A. 2019 Low-latitude zooplankton pigmentation plasticity in response to multiple threats. *Royal Society Open Science* 6: 190321. <http://dx.doi.org/10.1098/rsos.190321>

