Induced tolerance expressed as relaxed behavioural threat response in millimetre-sized aquatic organisms

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Natural selection shapes behaviour in all organisms, but this is difficult to study in small, millimetre-sized, organisms. With novel labelling and tracking techniques, based on nanotechnology, we here show how behaviour in zooplankton (Daphnia magna) is affected by size, morphology and previous exposure to detrimental ultraviolet radiation (UVR). All individuals responded with immediate downward swimming to UVR exposure, but when released from the threat they rapidly returned to the surface. Large individuals swam faster and generally travelled longer distances than small individuals. Interestingly, individuals previously exposed to UVR (during several generations) showed a more relaxed response to UVR and travelled shorter total distances than those that were naive to UVR, suggesting induced tolerance to the threat. In addition, animals previously exposed to UVR also had smaller eyes than the naive ones, whereas UVR-protective melanin pigmentation of the animals was similar between populations. Finally, we show that smaller individuals have lower capacity to avoid UVR which could explain patterns in natural systems of lower migration amplitudes in small individuals. The ability to change behavioural patterns in response to a threat, in this case UVR, adds to our understanding of how organisms navigate in the ‘landscape of fear’, and this has important implications for individual fitness and for interaction strengths in biotic interactions.

1. Introduction

Behaviour has important implications for individual fitness parameters and biotic interactions, and, accordingly, organisms are not passively staying in one habitat, but move to improve foraging and mating opportunities, or to avoid predators and harsh abiotic conditions [1,2]. Hence, evolution shapes behaviour, but behaviour can also change the path of evolution [2]. This has been well studied among large organisms, such as mammals, fishes and birds, which are easy to observe, for example by tagging them with various sensors that record their behaviour [3,4], showing that behaviour has important implications for a variety of ecological parameters, such as timing and duration of migrations, all affecting fitness [5,6]. However, similar studies on small (millimetre-sized) organisms are rare and constitutes a black box in behavioural ecology owing to methodological constraints, because it has been impossible to tag and track individual behaviour at this size range. However, with new, non-perturbing labelling and tracking techniques [7,8], we are here able to address the question of whether the behaviour of aquatic zooplankton (Daphnia magna) is affected by individual size, morphological traits and previous exposure to environmental selective forces, here exemplified with ultraviolet radiation (UVR).

Daphnia is a common millimetre-sized zooplankter genus in freshwater systems exerting strong grazing pressure on lower trophic levels. It is, for example, a key taxa in freshwater trophic cascades reducing the intensity of algal blooms.
Daphnia can however be sensitive to UVR and do suffer from higher mortality and DNA damage when residing in surface waters [10,11]. As a way of reducing damage, several different Daphnia species avoid UVR but at a cost of leaving warm and usually food-rich surface waters [12].

Behavioural responses in Daphnia have intrigued researchers for decades and changes in light intensity have been shown to initiate so-called diurnal vertical migrations ranging from 5 to 20 m in amplitude [13,14]. The adaptive benefit of diel vertical migration has traditionally been attributed to avoidance of visually hunting fishes [15], but Williamsson et al. [16] recently developed a more comprehensive framework where also other factors such as UVR exposure and food could explain some components of the behavioural pattern. UVR effects in aquatic systems have received more attention during the last couple of decades [17]. From being regarded as an unimportant factor owing to high attenuation in the water it has now been shown to have a variety of direct and indirect effects [10,17].

Regarding the actual mechanism by which Daphnia regulate their behaviour, it has been shown that the compound eyes are of vital importance for individual orientation [14]. Daphnia do have UVR-sensitive receptors in the compound eyes which are thought to help initiate the UVR-avoidance [18,19]. Also size of the individual animal has been suggested to affect the behaviour and small individuals display a lower amplitude during diel vertical migrations [20]. Juveniles also seem to be less responsive to UVR compared with adults [21].

Here, we study the behavioural response of large and small Daphnia that are either naive or used to the UVR threat. By rear- ing Daphnia for several months in UVR and non-UVR treatments and then exposing them again to UVR while studying their individual behaviour, we are here able to assess whether behavioural responses are correlated with individual size, eye size, pigmentation and/or previous experience to UVR. Moreover, we are also able to test the hypothesis that ‘training’ animals to UVR exposure will make them more adapted to handle the threat when again exposed to it, compared to naive siblings. Hence, we here add the response to an abiotic component—UVR—to the individual behavioural decisions in ‘the landscape of fear’, a concept previously mainly related to predator–prey dynamics [22]. Our study also opens up possibilities to understand the behavioural patterns of millimetre-sized organisms and the connection between behaviour, morphology and the level of adaptation to previously occurring selection pressures in the environment.

2. Material and methods

A population of D. magna was isolated from Lake Bysjön (55.6753 latitude, 13.5452 longitude) and was kept in the laboratory for several years (more than 100 generations) and fed at saturating levels with Scenedesmus sp. and under photosynthetically active radiation (PAR; Aura Ultimate Long Life 36 W; 12:12 D cycles). This source population was sampled and divided into two groups exposed to UVR and non-UVR treatment (denoted exposed and unexposed population, respectively). UVR exposure was produced by illuminating the containers with four UVA-340 Q-panel fluorescent tubes and four cool white fluorescent lamps (Q-panel UVA-340; Aura Ultimate Long Life 36 W). The UVR lamps produce a radiation spectrum which resembles the solar spectrum and are commonly used in UVR-exposure experiments [23]. UVR exposure was restricted in the unexposed population by covering this container with a UVR-screening Plexiglas, effectively cutting off radiation below 370 nm, i.e. in the UVA and UVB range (Röhm GS 233; Röhm, Darmstadt, Germany). UVR was allowed to enter the exposed population by covering this container with a UVR-transparent Plexiglas (Röhm GS 2458). However, transmittance of PAR was similar between Plexiglas types [24]. The intensity was 132 μW cm⁻² and 25.5 μW m⁻² s⁻¹ for UVA and PAR, respectively (SUL 033 and SUL 240; International Light, Newburyport, MA, USA; 12:12 D cycles). This approximately corresponds to a UVA dose similar to an autumn–winter day at temperate latitudes. The 1% attenuation depth for UVB was 1.0 ± 0.1 m (mean ± s.d.; i.e. depth where 1% of incoming radiation remains, estimated from absorption at 320 nm [25]). Daphnia were kept in this UVR and non-UVR treatment for eight months so that several generations would pass before the behavioural assays commenced. Individual Daphnia, both juveniles and adults, were then isolated from the two populations (exposed and unexposed) and kept in filtered water for 1 h prior to the behavioural assay. In order to be able to track their behaviour in three dimensions, we stained the animals with fluorescent nanoparticles according to methods developed by Ekvall et al. [8]. In short, fluorescent quantum dots (Life Technologies, Prod. no.: Q21321MP) were conjugated with poly-l-lysine which make them associate with the zooplankton. Daphnia were allowed to swim in 2 ml poly-l-lysine-conjugated quantum dots for 30 min. Most of the quantum dots solution was then removed and the Daphnia was allowed to swim in filtered water for a couple of minutes. After this standard staining procedure, individual Daphnia were introduced to the behavioural assay arena which was a 13.5 l Plexiglas aquarium (150 × 150 × 600 mm (L × W × H)) filled with 8.61 l of filtered water. The animal was allowed to acclimatize for 10 min under blue light and 20°C before the actual recording started (see Ekvall et al. [9] for standard settings). We then filmed the animal for 3 min under blue light, then turned on UVR for 3 min (0.1 A, corresponding to approx. 250 μW cm⁻²), and when UVR was switched off the animal was allowed to recover for 3 min. In all, this resulted in a 9 min long footage for each individual. For standard camera settings and radiation regime, see Ekvall et al. [8]. Each individual Daphnia was then tracked in three dimensions and the mean depth in the aquaria, the three-dimensional speed and the total distance travelled (i.e. the three-dimensional distance) were calculated according to standard methods in Ekvall et al. [8] and Bianco et al. [7]. Each size group (large and small) and population (exposed and unexposed) was replicated nine times (i.e. nine individual Daphnia). However, tracking was impossible in some individuals owing to reflections and bad image quality. Therefore, one replicate for large exposed, two replicates for large unexposed and two replicates for small individuals (both exposed and unexposed) were lost. For each individual Daphnia, we kept track of source population (exposed or unexposed) and we also measured body size (from centre of eye to the base of the spine) and eye diameter. We generally picked small and large individuals and the two size classes used were on average 2.7 ± 0.3 and 1.2 ± 0.2 mm (hereafter called large and small, respectively; mean ± s.d.). All size measurements were conducted using the ImageJ software [26] from pictures taken with a camera mounted on a stereomicroscope (Infinity 1–2 CB, Olympus SZ CTV; 20–40×; size measurement of the eye was impossible for one small individual owing to the handling procedure).

From the exposed and unexposed populations, we also isolated three samples per population with 30 adult Daphnia in each sample and analysed the melanin concentration of the animals. Melanin was extracted and analysed according to Hebert & Emery [27] and Hobaiter & Wolf [28]. The concentration of melanin pigments was normalized to dry mass calculated from published relationships of length to dry mass for Daphnia spp. [29].

Morphological traits were analysed with a two-way ANOVA and behavioural traits with a linear mixed model with size, population and UV (on/off) as fixed factors and individual as random
factor. All analyses were performed in R (v. 3.0.1; R Foundation for Statistical Computing, Vienna, Austria), and variables were log transformed when necessary to meet the assumptions of the tests.

3. Results

(a) Behaviour
Animals from both size classes (small and large) and treatments (exposed and unexposed to UVR, respectively) generally dwelled close to the surface during the first three acclimatization minutes (figure 1a). When UVR was switched on (min 4), there was a rapid response with downward swimming in both size classes and in both populations (figure 1a and table 1). There was also a size by UVR interaction indicating that large individuals had a slightly stronger UVR avoidance compared with small ones, and that small animals maintained a deeper depth distribution during the recovery phase (min 7–9), when the UVR-lamp was switched off (figure 1a and table 1).

Mean swimming speed was generally low during the acclimatization period (min 1–3), and then dramatically increased at UVR exposure (min 4–6; figure 1b). Following

![Figure 1. Behavioural responses of large and small Daphnia originating from populations reared in UVR (exposed) or reared in regular PAR-light (unexposed). Animals were first allowed to acclimatize for 3 min, then the UVR-lamp was switched on for 3 min (min 4–6, shaded box) and then on min 7 the UVR-lamp was switched off and animals were allowed to recover. Panels include: (a) mean depth, (b) speed and (c) total distance travelled. Points = median; line = mean; ×, outliers; top box = third quartile; bottom box = first quartile; whiskers = 1.5 times the interquartile range from the box.](http://rspe.royalsocietypublishing.org/issue/10.1098/rspb.2014.0364)
the initial peak, the speed levelled off and when the threat ended (min 7–9) the speed eventually returned to similar levels as before the threat (figure 1b). Interestingly, there were also differences between exposed and unexposed populations with slightly higher mean speed in the unexposed than the exposed population (figure 1b and table 1). Maximum speed also peaked during min 4, when UV was switched on and reached approximately 30 mm s\(^{-1}\) in large and 20 mm s\(^{-1}\) in small individuals. This UVR-induced behaviour led to a longer total distance travelled (i.e. three-dimensional distance) in large versus small \textit{Daphnia} and also a longer distance travelled in the unexposed population compared with the exposed population (figure 1c and table 1). The large unexposed animals showed a 9% longer total distance travelled than the large exposed individuals. On the other hand, small unexposed animals increased their total distance travelled with 46% compared with small exposed animals (figure 1c).

Table 1. Statistical results for behavioural responses of \textit{Daphnia} during 9 min trials. (Factors include size (large or small animal), population (populations either reared in UVR (exposed) or in regular PAR-light (unexposed), and UV (UVR or non-UVR exposure during the 9 min trial). Bold text indicates significant results.)

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\(p < 0.001\) and a population by size interaction (\(p = 0.003\)), emphasizing that small unexposed individuals displayed the strongest behavioural responses (see the electronic supplementary material with size normalized tests and figures).

(b) Morphology

The two size classes used were on average 2.7 ± 0.3 and 1.2 ± 0.2 long (large and small; mean ± s.d.), and there were no differences in length between exposed and unexposed populations (figure 2a and table 2). Eye diameter, normalized for body length, was smaller among large than small individuals (figure 2b and table 2). Moreover, animals coming from the exposed population had smaller relative eye diameter than unexposed animals (figure 2b). The amount of the photo-protective pigment melanin was similar between unexposed and exposed populations (0.15 ± 0.016 and 0.16 ± 0.013 \(\mu gDW^{-1}\), respectively; mean ± s.d.; table 2).

4. Discussion

A major aim in behavioural ecology is to understand how behaviour influences fitness parameters and ecological interactions, but also to assess to what extent behavioural responses are used to handle everyday threats. Moreover, in a continuously variable environment plasticity in behaviour is often crucial for individual performance and fitness [30]. Even though it is clear that behaviour is important, many conceptual
models do not include behaviour [31]. Moreover, available data to feed models are almost exclusively from large animals, which make it crucial to also provide behavioural knowledge for smaller sized animals.

Here, we demonstrate that size and previous selection pressures (exposure to UVR) affect individual behaviour and morphology of millimetre-sized animals. Exposure to UVR is a severe threat to zooplankton, leading to DNA damage and elevated mortality rates when dwelling in surface waters [10,11]. UVR is attenuated with depth [25] and will diminish as the animal swim deeper down. Hence, downward swimming is a way of rapidly avoiding otherwise detrimental radiation. Accordingly, the Daphnia in our study showed strong behavioural responses to UVR expressed in a rapid downward movement. But swimming downwards reduces the chance of feeding in warm and usually food-rich surface waters. This is an example of the ever continuing cost–benefit analysis that organisms have to perform to optimize their fitness. Similar types of trade-offs are common in feeding versus predator avoidance scenarios where for example squirrels alternate between open habitats where they gather food and then escape to safe habitats for feeding [32]. In our study all individuals, both small and large, from both exposed and unexposed populations (previous experience of UVR), exerted this immediate response to UVR. However, the strength in the response differed among individuals and between animals previously exposed or unexposed to the threat. This suggests that also small, millimetre-sized, animals are able to make individual, context-dependent decisions in a ‘landscape of fear’ in a similar way as mammals acting in a predator–prey context [22].

Large individuals were faster and swam longer total distances in response to UVR compared with small individuals. For example, a 2.7 mm long individual travelled on average 500 mm per minute, whereas a 1.2 mm animal travelled 220 mm during the same time. If the total distance travelled is normalized to body length, then size effects disappear indicating that small Daphnia were avoiding UVR at their maximum capacity. Small Daphnia have previously been shown to display lower amplitude during diel vertical migrations [20] and also seem to be less responsive to UVR compared with adults [21]. Our detailed tracking study shows that smaller individuals are travelling at lower speed and also travel shorter distances compared with larger individuals which offers an explanation to the patterns observed in previous studies.

Moreover, the UVR-exposed population was in general less responsive to UVR compared to the naive, unexposed population. The unexposed small animals displayed the strongest response with 46% longer distance travelled compared with the exposed small animals. This strongly suggests that exposure to UVR induced elevated tolerance to the threat. A potential explanation to this tolerance may have been increased amounts of photo-protective pigmentation [24,33]. The major pigment in Daphnia is melanin, and it has previously been shown that populations with high melanin pigmentation show little behavioural response to UVR [12,24]. Highly melanized Daphnia populations are common in clear high altitude and latitude ponds [27], which may explain the small variation in pigmentation of the present population which originates from a lowland, temperate lake. Morphological and behavioural traits in general are often connected, for example, vertical migration and the level of protective pigmentation [24,34]. Trait compensation is common in a variety of organisms for example in snails where individuals with little morphological defences display a greater behavioural avoidance of predators and vice versa [35]. However, in our study, the melanin concentrations were low and did not differ between unexposed and exposed animals, suggesting that pigment level was not a plastic trait and that induction of photo-protective melanins was not causing the more relaxed response to UVR among previously exposed individuals. Apart from UVR-avoidance, many zooplankton taxa can also display other UVR defences such as photoenzymatic repair and the induction of internal antioxidants [36,37], which may help non-pigmented animals to survive UVR exposure.

Although it is unknown which of these UVR defences improved the tolerance to UVR in our exposed animals, the UVR-sensitive organelles are situated in the compound eye of Daphnia [18]. Interestingly, individuals from the UVR-exposed population had smaller eye diameters than unexposed animals. Factors changing the pigmentation and size of the eye are not well understood but the pigmentation of the eye varies with light regime [38]. These daily oscillations in eye pigmentation are suggested to be a sign of an internal clock regulating behaviour [38]. Although it was beyond the scope of this study to assess whether eye size differences affected the behaviour of the Daphnia, or if eye differences were a mere effect of UVR exposure decoupled from the behavioural response, we conclude that small eye diameter was associated with UVR exposure.

It can also be difficult to extrapolate these results to natural populations. The present population had been cultivated in the laboratory for many years which may erode the genetic diversity [39]. If there were only a few clonal genotypes, then we may expect hampered natural selection. Thus, the relaxed sensitivity to UVR by the exposed group was induced, but whether or not this is an adaptive response is still an open question.

In conclusion, we here demonstrate that UVR-avoidance was immediate and present in all size classes studied, but that small individuals were travelling shorter distances compared with larger ones. Individuals pre-exposed to UVR showed a more relaxed response to the UVR threat than naive
References


