Individual changes in zooplankton pigmentation in relation to ultraviolet radiation and predator cues

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Abstract
Copepods are common crustaceans in aquatic systems and one of the most important producers of carotenoid astaxanthin pigments, which can enhance the animals’ resistance against potentially damaging ultraviolet radiation (UVR), but at the same time, increases the risk of fish predation. Previous studies have demonstrated that copepods have different pigmentation levels matching the current threat level in terms of UVR and fish occurrence. However, these other studies have quantified population-levels changes in pigmentation, making it difficult to disentangle the role of individual phenotypic colour changes from that of selection. We quantified carotenoid-based pigmentation with colorimetric methods, which enabled us to track changes within individual copepods. Two species of copepods, *Diaptomus castor* and *Eudiaptomus gracilis*, were exposed to high and low UVR and fish cues in a factorial design. L*a*b* colour values (CIE; Commission International de l’Eclairage) were extracted from digital photographs of each copepod and used as proxies for carotenoid concentration. Our results showed that individual copepods significantly changed their pigmentation in response to both UVR and fish cues within a period of 2 weeks. However, the responses differed between sexes and between adults and juveniles. UVR effects were present in all life-stages whereas fish effects were only detected in juveniles, with largest responses in *D. castor*. This confirms that carotenoid pigmentation is a phenotypically plastic trait, and highlights that strategies for trading off risks of UVR and predation differ between males and females as well as between life-stages.

Carotenoid-based pigmentation is ubiquitous in nature and serves many different functions, such as in photoprotection, signalling, and thermoregulation (Wong and Svensson 2011). In copepods, which are common aquatic zooplankton, populations vary in their carotenoid pigmentation (Haitson 1979a). Highly pigmented populations are typically found at high latitude and altitude sites, but in certain cases also in lower latitude systems (Mani 1962; Needham 1974; Luecke and O’Brien 1981; Jarvis 1988). The carotenoid astaxanthin is the main pigment in copepods and is produced in the animal with help of precursors from the algal food source (Haitson 1979a; Bandaranayake and Gentien 1982; Miki 1991; Matsuno 2001). This pigment has been demonstrated to give protection against intense light and ultraviolet radiation (UVR) damages (Haitson 1979a; Byron 1982; Miki 1991) by reducing the concentration of free radicals that otherwise would damage vital bio-molecules (Miki 1991; Woodall et al. 1997; Caramujo et al. 2012). Populations with lower pigmentation suffer from higher UVR-induced mortality compared with populations with higher pigmentation (Moeller et al. 2005).

The threat of UVR damage is generally highest in spring and summer months (Hansson 2004), which should lead to the highest pigmentation during this time. However, fish and other predators have been shown to target heavily pigmented individuals of zooplankton (Haitson 1979b; Luecke and O’Brien 1981) and in lakes devoid of fish, zooplankton have higher pigmentation compared with the ones in lakes with fish (Hansson 2000). Hansson (2004) suggested that individual copepods can optimize their pigmentation level to match the current threats from both UVR and fish predation. This leads to a prediction for high pigmentation during winter and early spring, and low during summer (Hansson 2004; Snoeij and Häubner 2014). Changes in pigmentation in a population occur over a time scale of a few days, and experiments have verified that populations reduce carotenoid-based pigmentation when exposed to fish cues, and increase levels if exposed to UVR (Hansson 2000, 2004). One limitation of studying small-bodied animals like copepods is the reliance on samples containing many individuals. Previous studies have quantified pigmentation with destructive sampling of pooled samples of
a large number of individuals. This approach has two issues. First, as individuals are not followed over time, it is hard to disentangle phenotypically plastic changes from population-wide responses, such as selection. Second, it limits the potential to detect differences in pigmentation strategies between different individuals, during ontogeny and between males and females. To be able to determine if changes in pigmentation occur at the individual level, we therefore developed a technique to quantify pigments non-destructively using colorimetric methods. We did this in two different calanoid copepod species (Diaptomus castor and Eudiaptomus gracilis), before and after exposure to both UVR and fish cues in a factorial design. D. castor originated from a shallow clear-water pond without fish predators and E. gracilis from another clear-water lake with some fish predators. Body size and life stage may affect both predation risk and the susceptibility of tissue damage from UVR (Brooks and Dodson 1965; Hylander et al. 2014). Therefore, we predicted that males, females and juveniles would have different strategies to address the trade-off between photoprotection and predation avoidance. In particular, we expected to find within-individual changes in pigmentation in response to both UVR and fish cues, but also an interaction effect between these two factors since the degree of UVR-induced pigmentation could depend on the level of predation threat.

**Material and methods**

**Experiment setup**

*D. castor* was collected from a shallow (< 1 m) fish-free pond on Oland, Sweden (56°36′30.06″N, 16°29′44.61″E). The mean body length was 2.4 mm, including the prosome and urosome, excluding cadal satae. *E. gracilis* was collected from Dalby quarry (55°39′32.76″N, 13°24′4.87″E; mean body length: 1.2 mm), a water body with a maximum depth of 6 m and containing a sparse fish population of rainbow trout (Onchorhyncus mykiss).

A total of 60 copepods were randomly isolated from each location and were placed in separate glass beakers (Diameter 8 cm, Height 7 cm) with 200 mL of 0.2 μm filtered water. To minimize handling, sex and/or life-stage were not determined at this stage of the experiment. Scenedesmus obliquus was used as the food source at a concentration of 30,000 cells mL⁻¹, because of its documented high level of astaxanthin precursors (i.e., β-carotene) (Qin et al. 2008).

The 60 glass beakers were then randomly split in four groups according to a factorial design and placed 45 cm directly under two fluorescent UV-lamps (Q-panel UVA-340; Aura Ultimate Long Life 36W) on a 12 : 12 light to dark cycle and a UVA irradiance of 400 μW cm⁻². The lamps are commonly used in UV-exposure experiments and have a spectrum that resembles the solar spectrum in the UV-wavelength range from 300 nm to 400 nm except for lower levels of long wave UVA radiation (Moeller et al. 2005; Hansson et al. 2007). The first factor was UVR-exposure, where one treatment had beakers covered with UVR-transparent acrylic filters (Röhm GS 2458; Röhm, Darmstadt, Germany, average transmittance 85% between 300 nm and 400 nm), and the other beakers had UV-blocking filters (Röhm GS 233) that effectively remove UVA and UVB by blocking radiation below ~ 370 nm (< 1% transmittance between 300 nm and 370 nm, 50% transmittance at 379 nm and 90% at 405 nm). Transmittance of photosynthetically active radiation (PAR) is similar for the two types of filter (Hansson et al. 2007). The second factor was exposure to fish cues. Every day each beaker received 1.5 mL of frozen water. The fish cue treatment received filtered water (Whatman, GF/F) from an aquarium containing a mix of bleak (Alburnus alburnus) and roach (Rutilus rutilus) (600 L tank with 100 fishes). The no-predator treatment received filtered water from an aquarium without any fish present.

Copepods were incubated for 14–15 d (*D. castor*: from 19 November 2014 to 04 December 2014; *E. gracilis*: from 18 March to 01 April). After 1 week, 100 mL of the water was exchanged to ensure good water quality and *S. obliquus* concentrations were checked and adjusted to the initial concentration.

**Photography and colorimetry**

A digital photograph was taken of each copepod at the start and end of the experiment (15 d and 14 d for *D. castor* and *E. gracilis*, respectively). The images were obtained with a Canon EOS 700D camera, mounted on a microscope (Olympus SZX7). The images were taken in a dark room with only one light source (Photonic, High-Power LED spots) mounted on a stand in a 45° angle 7 cm from the copepod, with the camera set on “manual” mode (ISO 200, Shutter speed 1/80s). Great care was taken to standardize the conditions at image capture. (Fig. 1)

The images were imported to the software (Photoshop CS6 © 2015 Adobe Systems Incorporated) and converted to Photoshop Lab colour, which is based on the CIE (Commission Internationale de l’Eclairage) L*a*b* colour space. CIE L*a*b* is a standardized, uniform and device independent colour space that separates luminescence (L*: from dark to light) from two measures of colour intensity, a* and b* (Chen and Hao 2004). In Photoshop Lab, a* = 0 describes what to human eyes is pure green, while 128 describes neutral grey and 255 describes pure magenta. For b*, 0 describes pure blue, 128 describes neutral grey and 255 describes pure yellow. The a* and b* channels have been successfully used as proxies for carotenoid-based coloration (Svensson et al. 2005). Although chromatography is required to separate and identify individual carotenoids, a* (“redness”) correlate well with the concentration of relatively red carotenoids, such as astaxanthin (Hatlen et al. 1998), while b* (“yellowness”) correlate better with more yellow carotenoids, such as lutein (Humphries et al. 2004). The precise relationship between CIE measurements and carotenoid concentration varies between organisms. However, the
relationships are positive (linear or curvilinear), and $a^*$ and $b^*$ typically explain 50–90% of the variation in carotenoid concentration (Hatlen et al. 1998; Humphries et al. 2004; Svensson et al. 2005). To avoid measurement noise from green gut content, only the average colour values of the cephalosome was selected for the analysis. Copepods can contain multiple pigments (e.g., astaxanthin, astaxanthin esters, and canthaxanthin) that may differ in hue (Rautio et al. 2009; Snoeijis and Häubner 2014) and we therefore chose to quantify both $a^*$ and $b^*$ channels as proxies for overall pigment concentration.

To account for individual changes in pigmentation we quantified $a^*$ and $b^*$ values in each individual 14–15 d after exposure to the treatments, depending on species and subtracted from the initial values before exposure (Fig. 1). This gave us a value describing the change in pigmentation in individual copepods during the treatment (positive $a^*$ and $b^*$ values indicate increases in red and yellow pigmentation, respectively).

Determination of copepod life-stage (C1–C6) was not possible without killing the animals. We instead divided the copepods into adult males, adult females and juveniles using the photographs. Individuals were defined as adults based on size and occurrence of well-developed sex-specific morphology such as outgrowths on 1st antennae and hyaline spines on thoracic wings. Among adults, females of both species had elongated thoracic segments and males had outgrowths on the 1st antennae. Since sex determination can be difficult in copepodes (even though outgrowth on 1st antennae was visible in some late stage copepodites), all juvenile stages were treated as one group. In all, there were 14 and 4 males, 25 and 26 females, and 21 and 30 juveniles among $D. castor$ and $E. gracilis$, respectively. One way ANOVAs with Tukey tests were used to determine initial difference in pigmentation between life-stages. The results of the experimental treatments were analysed as fully factorial models with UVR, predator cues and the interaction UVR : predator cues as fixed factors. Models with non-significant interactions were re-fitted without these terms ($z = 0.05$; according to Crawley 2007). In models with significant interactions, data were split and the effect of predator cues was analysed separately in each of the two UVR treatments. To reduce the risk of spurious interactions we chose to perform separate analyses of males, females, and juveniles, as well as of the two species. All statistical tests were performed in R 3.0.2 (R Core Team 2013).

**Results**

**Initial variation in pigmentation within populations**

There were some initial differences in mean pigmentation among adult males, females, and juveniles in both species...
The cephalosome of *D. castor* males had an overall higher $a^*$ value (i.e., stronger red colour) compared with the females (Tukey's tests, $p < 0.004$). There was also a nonsignificant trend for males to have higher $a^*$ than juveniles (Tukey's test, all $p > 0.098$). However, there were no differences in initial $b^*$ values (Tukey's test, all $p > 0.48$). Among *E. gracilis*, juveniles had higher $a^*$ pigmentation compared with females (Tukey's tests: $p = 0.029$). There was also a non-significant trend for more $b^*$ pigmentation in juveniles compared with females (Tukey's tests, $p = 0.062$). There were no other differences in the initial coloration in this species (Tukey's test, all $p > 0.37$). Due to the low number of *E. gracilis* males, statistical comparisons with juveniles or females were not possible.

**Changes in individual pigmentation in response to UVR and fish cues**

Individual copepods significantly changed their cephalosome pigmentation over the course of the experiment. However, the responses to the treatments differed between species, sexes and life-stages. *D. castor* males responded to UVR exposure with increased pigmentation in both $a^*$ and $b^*$ colour channels (Table 1; Fig. 1 and 3). Male *D. castor* did not respond to predator cues, and females did not respond to either of the treatments. Juvenile *D. castor* showed a complex response, because there was a significant interaction between predator and UVR treatment, in the $b^*$ channel only. This indicates that the change in yellow pigmentation in juveniles depends on both predator cues and UVR exposure. Investigating this interaction further, we found that the juveniles in non-fish treatments reduced yellow pigmentation less in UVR compared with non-UVR treatments. However, when predator cues were present, they reduced their yellow pigmentation regardless of UVR-exposure (Table 1; Fig. 3). Juveniles of *D. castor* that were exposed to UVR but not exposed to fish cues maintained more yellow cephalosome pigmentation (higher $b^*$-value) compared with all other treatment combinations.

*E. gracilis* showed slightly different patterns compared with *D. castor*. *E. gracilis* females increased the redness ($a^*$ channel) of their cephalosome if they were exposed to UVR (Table 1; Fig. 3). In Juvenile *E. gracilis* UVR had a similar effect on the $a^*$ channel and possibly also the $b^*$ channel (Table 1), although caution should be exercised when interpreting $p$-values close to 0.05 when having fit several models to the data. As there were too few *E. gracilis* males for statistical analysis, we cannot rule out that males may have also responded to the treatments. Predator cues seemed to have little effect on *E. gracilis* copepods: there were no significant main effect of cues and no interaction with the UVR treatment.

**Discussion**

Individual phenotypic changes vs. population wide responses such as selection are difficult to separate, especially among small organisms. It has for example been proposed that zooplankton can phenotypically increase their pigmentation in response to UVR to reduce harmful damages caused by radiation (Hansson 2000, 2004). Analytical methods used so far to quantify pigmentation require pooled samples with many individuals since the animals are in the mm-range (Hansson 2000; Hansson et al. 2007; Persaud et al. 2007; Rhodes 2007; Hylander et al. 2009). Hence, previous results, albeit intriguing, have not been able to determine if pigmentation changes were in fact due to individual plasticity or population wide responses such as selection. The strength of our study is that using colorimetry, we were able to study changes in individual copepods. Moreover, by separating males, females, and juveniles, we were able to detect differences in how these groups responded to UVR and predator cues.

Our new colorimetric method demonstrated that there was a large natural variation in pigmentation among...
individuals both within and among sexes and life-stages (males, females, juveniles). For example, adult males of *D. castor* had more red pigmentation in their cephalosome compared with females. The underlying reasons for sex and life-stage differences are unknown but life-stage specific pigmentation suggests different utilization of the pigment during the development. Males, females, and juveniles are likely to differ in how they optimize carotenoid allocation. For example, the main carotenoid in copepods is astaxanthin which is a strong antioxidant that can neutralize harmful reactive oxygen species (Hairston 1979a; Byron 1982; Miki 1991; Hansson 2000). In general, pigmented populations are therefore more resistant to intense light and UV-radiation compared with transparent populations (Hairston 1976; Moeller et al. 2000).

| Response | Life-stage | Factor         | Sum Sq | Df1 | Df2 | F value | p      |
|----------|------------|----------------|--------|-----|-----|---------|--------|
| **Diaptomus castor** | a* | Adult female | UVR | 15.36 | 1 | 6 | 0.41 | 0.547 |
| | Predator | 130.19 | 1 | 6 | 3.44 | 0.113 |
| | UVR:P| 6.69 | 1 | 5 | 0.15 | 0.713 |
| | Adult male | UVR | 1026.03 | 1 | 11 | 94.97 | <0.001 |
| | Predator | 5.16 | 1 | 11 | 0.48 | 0.504 |
| | UVR:P| 18.20 | 1 | 10 | 1.81 | 0.208 |
| | juvenile | UVR | 23.96 | 1 | 12 | 0.98 | 0.343 |
| | Predator | 27.86 | 1 | 12 | 1.14 | 0.308 |
| | UVR:P| 2.13 | 1 | 11 | 0.08 | 0.782 |
| | b* | Adult female | UVR | 151.50 | 1 | 6 | 1.21 | 0.314 |
| | Predator | 211.53 | 1 | 6 | 1.69 | 0.242 |
| | UVR:P| 70.45 | 1 | 5 | 0.52 | 0.504 |
| | Adult male | UVR | 780.76 | 1 | 11 | 20.10 | <0.001 |
| | Predator | 16.09 | 1 | 11 | 0.41 | 0.533 |
| | UVR:P| 43.13 | 1 | 10 | 1.12 | 0.314 |
| | juvenile | UVR | 1027.92 | 1 | 11 | 29.92 | <0.001 |
| | Predator | 12.82 | 1 | 11 | 0.37 | 0.554 |
| | UVR:P| 314.89 | 1 | 11 | 9.16 | 0.012 |

Data split according to predator treatment

| Factor | Sum Sq | Df1 | Df2 | F value | p      |
|--------|--------|-----|-----|---------|--------|
| UVR (predator cues) | 26.81 | 1 | 3 | 0.42 | 0.560 |
| UVR (no predator cues) | 1316.00 | 1 | 8 | 55.88 | <0.001 |

| **Eudiaptomus gracilis** | a* | Adult female | UVR | 16.13 | 1 | 8 | 8.12 | 0.022 |
| | Predator | 3.98 | 1 | 8 | 2.00 | 0.195 |
| | UVR:P| 0.01 | 1 | 7 | 0.00 | 0.969 |
| | Adult male | UVR | # | # | # | # |
| | Predator | # | # | # | # |
| | UVR:P| # | # | # | # |
| | Juvenile | UVR | 2.73 | 1 | 16 | 9.08 | 0.008 |
| | Predator | 0.012 | 1 | 16 | 0.04 | 0.846 |
| | UVR:P| 0.55 | 1 | 15 | 1.94 | 0.184 |
| | b* | Adult female | UVR | 0.05 | 1 | 8 | 0.00 | 0.972 |
| | Predator | 25.86 | 1 | 8 | 0.62 | 0.452 |
| | UVR:P| 43.41 | 1 | 7 | 1.06 | 0.338 |
| | Adult male | UVR | # | # | # | # |
| | Predator | # | # | # | # |
| | UVR:P| # | # | # | # |
| | Juvenile | UVR | 233.17 | 1 | 16 | 4.64 | 0.047 |
| | Predator | 3.40 | 1 | 16 | 0.07 | 0.798 |
| | UVR:P| 100.44 | 1 | 15 | 2.14 | 0.164 |

Table 1. Results from factorial models analysing the effect of UVR treatment and predator cues on changes in the pigmentation (a* red and b* yellow) of individual copepods. See main text for details. *E. gracilis* male insufficient data for statistical analysis (#). Bold font indicates significant results.
It has recently been shown that copepods with a higher RNA: DNA ratio are also more pigmented, suggesting a growth benefit of the pigment (Gorokhova et al. 2013). High carotenoid levels could therefore carry extra benefits in rapidly growing individuals. Furthermore, females face an additional allocation trade-off, as large amounts of carotenoids often are deposited into the eggs (Hairston 1979a). The physiological requirements of a high somatic growth and of egg production can explain our finding that males, females, and juveniles differ both in their initial pigmentation and in how they respond to threats from predation and UVR damage. Some caution is required when interpreting differences in cephalosome pigmentation between sexes and life stages. For example, mature females often transfer pigments to eggs (Hairston 1979a), which may bias the pigmentation estimate if only analysing the cephalosome. To ensure that our results were robust, we also analysed the entire prosome in males, females, and juveniles. This analysis showed the same patterns, although the effects were weaker, most likely due to noise caused by variation in the amount of green gut content.

An intriguing question is whether zooplankton pigmentation is a phenotypically plastic trait. This has been suggested from studies using pooled samples of many individuals (Hansson 2000; Hansson et al. 2007; Persaud et al. 2007; Rhodes 2007; Hylander et al. 2009). Here, we demonstrate that copepods on an individual level changed their pigmentation in response to UVR and that the response can occur within 2 weeks. Phenotypic plasticity is generally favored in environments that are variable and where expressing the traits has some cost to the individual (Tollrian and Heibl 2004; Gorokhova et al. 2013). UVR irradiance fluctuates heavily over a day depending on the weather condition, e.g., cloud cover (Hansson et al. 2016). At our sampled sites, the irradiance also varies by a factor 10 seasonally (Ekvall et al. 2015) and inducible defences in response to this environmental factor could be beneficial. Highly pigmented individuals are easily spotted and predation on pigmented animals is higher than on more transparent conspecifics (Hairston 1979b; Luecke and O’Brien 1981). Copepod populations in lakes without fish do generally have higher pigmentation compared with lakes with fish (Hansson 2000). Hence, there would be a trade-off between UV-protection with high pigmentation and ability to escape predation with transparency (Hansson 2004). In addition, there could be metabolic costs to convert plant carotenoids to astaxanthin (main carotenoid in copepods). We did not observe any pigmentation changes in response to predator cues among adult animals. Juveniles, however, did respond to UVR when fish cues were absent but there was no such response when fish cues were present.

Fig. 3. Differences in pigmentation (mean ± SE) from initial values in males, females, and juveniles in *D. castor* and *E. gracilis* in the different treatments (No-UV, UV, No-UV + fish, and UV + fish). Gain in pigments displays as positive values and loss in pigments displays as negative values.

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**Fig. 3.** Differences in pigmentation (mean ± SE) from initial values in males, females, and juveniles in *D. castor* and *E. gracilis* in the different treatments (No-UV, UV, No-UV + fish, and UV + fish). Gain in pigments displays as positive values and loss in pigments displays as negative values.
present. This suggests a reduced production and/or accumulation of pigments during their development if there is a threat of fish predation. Hence, physiological changes in pigmentation in response to fish predators could be restricted to the growth and development phase of the copepod. The reason for such life-stage (juvenile vs. adult) specific pigmentation response is not known but one could speculate that it has to do with differential trade-offs in energy allocation during ontogeny. Sex specific trade-offs have for example been shown to lead to differential behaviour (and energy allocation) among male and female copepods (Ceballos and Kiorboe 2011).

Life-stage and sex-specific phenotypic changes in pigmentation are intriguing and suggest different selective forces acting within a population. For example, adult males of D. castor responded to UVR whereas females did not, and the juveniles responded mainly by maintaining high pigmentation in a high-UVR/low predation scenario. Juveniles for both species had a higher yellow pigmentation and lower red pigmentation compared with males of the same species (Fig. 2). This indicates life-stage specific differences in the type of carotenoids that are present. Based on previous studies, a high a* (redness) in the adults could derive from high concentrations of the astaxanthin, since it is produced from β-carotene and is the main carotenoid found in crustaceans (Rhodes 2007). While juveniles were more yellow, possibly due to lower levels of astaxanthin or a different mixture of astaxanthin and its esters compared with the adults. However to distinguish between individual carotenoids is not possible by colorimetry alone, and future studies using chromatography are needed to investigate the exact pigment blend. All copepods received the same food source (S. obliquus) and this phytoplankton species is known to be rich in the precursors (β-carotene and zeaxanthin) (Qin et al. 2008) which is required when zooplankton produce and accumulate astaxanthin (Rhodes 2007). Hence, it is unlikely that the astaxanthin precursors were limiting in the food supply.

Conclusion

We can confirm that changes in pigmentation do occur at the individual level of this mm-sized crustacean in response to exposure to UVR and to threat from predation. Interestingly, our method was able to identify that both ontogeny and sex influence these responses. Up until now these patterns have been unknown, due to methodological constraints. Variation in how selective forces act on different life-stages in mm-sized animals is an intriguing topic in ecology. Individual-based studies can open up new avenues in our understanding of natural selection in the microscopic world.

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