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ORIGINAL PAPER

Pigmentation and spectral absorbance in the deep-sea arctic amphipods *Eurythenes gryllus* and *Anonyx* sp.

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Abstract As for many deep-sea animals, the red colouration of the two amphipods *Eurythenes gryllus* and *Anonyx* sp. has an important function providing camouflage, as the attenuation of the red wavelengths in seawater is higher than other colours within the visible range. Variation in colouration between different stages of colour intensity (related to size) is evident in both species. The red colour is caused by carotenoids, and the carotenoid composition was identified and quantified using spectral optical density signatures, high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS). The carotenoid astaxanthin was identified as the major carotenoid in both amphipods, both in pure and in esterified forms. In addition, minor amounts of lutein-like, canthaxanthin-like and several unidentified carotenoids were found in *E. gryllus*, while diatoxanthin, β,β-carotene and canthaxanthin-like carotenoids were detected in *Anonyx* sp. Generally, both species displayed an increase in the amount of carotenoids as a function of colour intensity and size. Shifts in λ_{max} in the OD (Optical density; dimensionless, acronym absorbance) spectra were evident in both species between the different colour stages in both the in vivo and the in vitro material, probably caused by changes in pigment composition. Similar shifts in λ_{max} were observed between the in vivo and in vitro pigment raw extracts in general, most likely caused by pigment-binding proteins. The differences in pigment composition and wavelength shifts suggest large intra- and inter-specific differences between the two species. Probable reasons for changes in pigment composition could be related to diet, season, moulting patterns, metabolic pathways and reproduction.

Keywords Carotenoids · In vivo absorbance · Deep-sea · Amphipods · Camouflage · Arctic

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Introduction

The red and orange colouration of many organisms and especially within the Crustacea are often caused by carotenoids, a group of fat-soluble pigments which are synthesized de novo in higher plants, mosses, bacteria, algae and fungi (Goodwin 1980; Gaillard et al. 2004), thereby making it available for other organisms through their diet. Carotenoids have various important biological functions regarding vision (Vershinin 1999), ovarian maturation (Dall et al. 1995), reproduction (Gilchrist and Lee 1972) and development (Petit et al. 1998; Berticat et al. 2000). One of the most common carotenoids in marine invertebrates is astaxanthin, which belongs to the xanthophylls and is usually formed through oxidative transformation from ingested β,β-carotene or zeaxanthin (Katayama et al. 1971; Tanaka et al. 1976). Astaxanthin has received a great deal of attention in the last couple of decades, due to its ability to act as an antioxidant and immunostimulant and thereby potentially play a role in the fight against human illnesses like cancer and cardiovascular diseases (Fraser and Bramley 2004; Higuera-Ciapara et al. 2006; Cornet et al. 2007).

While one of the important functions of carotenoids in shallow water organisms is to prevent photodamage caused by solar radiation (Hairston Jr 1976; Bidigare et al. 1993; Schubert and García-Mendoza 2008), this obviously has no function in the deep-sea. However, carotenoids do serve an important function in the deep-sea in terms of camouflage (Herring 1972). Due to the exponential attenuation of sunlight with increasing depth, red wavelengths (~650 nm) being the first to disappear and blue wavelengths (~490 nm) reaching depths around a 1,000 m, a uniform distribution of red pigmentation in the exoskeleton of a deep-sea crustacean would provide valuable camouflage against predators (Herring 2002; Robison 2004; Johnsen 2005) both with regard to scattered surface light and by preventing reflection from bioluminescent light, which is a common trait in many deep-sea predators (Nicol 1958; Herring 1972).

Deep-sea scavenging amphipods (*Crustacea, Malacostraca*) feeding on carrion and detritus (Sainte-Marie 1992; Smith and Baco 2003) play an important role in the dynamics of the deep-sea food web by contributing to the decomposition and redistribution of large food falls (Hessler et al. 1978; Hargrave et al. 1994). One such scavenger is the necrophagous (feeding on carrion or corpses) lysiannassoid amphipod *Eurythenes gryllus* (Stoddart and Lowry 2004), which is one of the best studied species of deep-sea amphipods to this time. It is recorded in all major oceans (except the Mediterranean Sea), has a bentho-pelagic lifecycle, is found at depths down to 7,800 m (Ingram and Hessler 1983; Ingram and Hessler 1987; Thurston et al. 2002; Stoddart and Lowry 2004) and is a common member of the benthic mega-fauna found in deep-sea communities. Another lysiannassoid amphipod genus found in the deep-sea is *Anonyx*, which consists of mostly necrophagous scavengers which usually share the same role in the benthic community as *E. gryllus,* but are often found in shallower waters (Ingólfsson and Agnarsson 1999; Werner et al. 2004; Legeżyńska 2008). Both *E. gryllus* and *Anonyx* spp. occur in a range of colours, from white through different shades of orange and pink to a dark red colouration (Smith and Baldwin 1984). Earlier studies of *E. gryllus* have indicated an increase in colour intensity with age and sexual maturation (Smith and Baldwin 1984; Charmasson and Calmet 1987), but further research has yet to be published on this topic. Some *Anonyx* species, such as *A. nugax* (Obermüller et al. 2005), have a yellow or orange colouration, although this may vary between individuals. While the greatest species diversity of *Anonyx* occurs in the North Pacific (Steele 1979), which is also its place of origin, Arctic and North Atlantic waters show a much lower species diversity, probably due to slow dispersion. The morphological similarity of the species in this genus makes them difficult to identify, and many species have remained unrecognized for a long time (Steele 1979).

Most of the carotenoids found in deep-sea animals originate in phytoplankton in the surface layer and are subsequently transferred down through the food web (Herring 1972) or as suspended particulate matter containing unaltered carotenoids as reported by Repeta and Gagosian (1984) in 1,500 m depths off the Peruvian coast.

Although a few larger species have been known to act as predators, attacking weak or vulnerable prey items, most deep-sea amphipods are usually regarded as scavengers on detritus or carrion, which would most likely be where they obtain their carotenoids (Ingram and Hessler 1983; Smith and Baldwin 1984; Sainte-Marie and Lamarche 1985). Sainte-Marie and Lamarche (1985) found that among large *Anonyx* spp., normal food items included carrion, large copepods such as *Calanus,* which is an abundant genus in Arctic waters (Falk-Petersen et al. 2009), polychaetes and small crustaceans including euphausiids, mysids, cumaceans and amphipods. Wlodarska-Kowalczuk et al. (2004) reported a higher biomass of macrofauna in the ice-free areas outside Kongsfjorden in Svalbard, compared to other areas in the Arctic basin which were covered by multi-year ice, with a dominance of polychaetes and bivalves together with several species of crustaceans. This indicates a high availability of food items for amphipods in this area, either as carrion or as prey, and hence a reliable source of carotenoids.

The main aim of this study was primarily to identify and quantify the pigment composition in *E. gryllus* and *Anonyx* sp. Additionally, we aimed to explore the relationship between size, pigmentation and the corresponding in vivo and in vitro spectral absorbance characteristics within each species as well as any interspecific differences.

Materials and methods

The samples were collected at approximately 1,200 m depth, off the continental shelf outside the entrance of Kongsfjordrenna (79° 04.87′N; 007° 52.72′E) in West-Spitsbergen, Svalbard (Fig. 1) during two cruises with R/V "Jan Mayen", the first trap deployment at 19th of August 2007 (traps on seafloor for 6 days) and the second trap deployment at 23rd of August 2008 (traps on seafloor for 4 days).

The traps were constructed using PVC tubes converted into traps by placing an inverted funnel in one end and a mosquito net in the other end. The traps were attached to a frame and filled with dead polar cod (between 4 and 10 polar cod per trap) before they were deployed. Recovery of the traps was carried out by the help of an acoustic releaser (IXSEA, Oceano 2500) and flotation buoys.

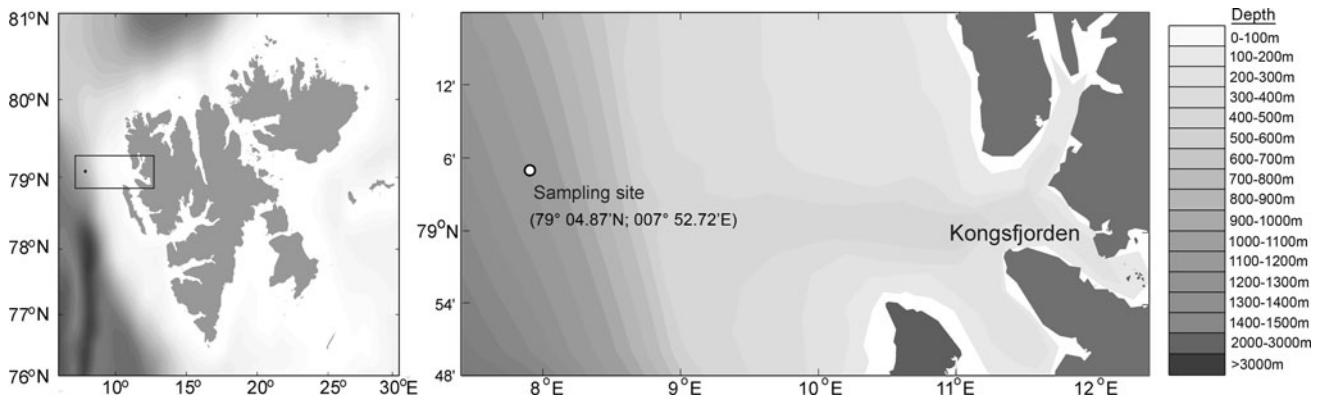

Fig. 1 Map over Konsfjordrenna, Svalbard

The temperature at 1,200 m depth was about −0.8°C and the salinity at 34.9 PSU, indicating cold Atlantic water.

The collected material comprised two species, *Eurythenes gryllus* and *Anonyx* sp. The former is a cosmopolitan deep-sea species previously recorded from both the Arctic and Antarctic (Premke et al. 2003; De Broyer et al. 2004; Stoddart and Lowry 2004), whereas the latter is a species known only from the Svalbard region that has yet to be described (Berge, personal communication). Unfortunately, due to a clean-up in a biofreezer, approximately 3/4 of the collected specimens of *E. gryllus* was lost. The rest of the specimens were then size-fractioned according to the following description.

The two species were split into discrete colour groups as a compromise between sample size and information about colour intensity (Figs. 3, 4). In addition, the body length (in cm) of each amphipod from each colour stage was recorded. One-way analysis of variance (one-way ANOVA) and Tukey's HSD (honestly significant differences) were used for the statistical analysis.

The shell and/or tissue used for extraction were weighed using a Sauter AR 1014 weight with ±0.001 g accuracy before measurements were taken. Optical density was used to describe spectral differences from 350 to 700 nm in vivo and in vitro. The term "absorption" (m^{-1}) is only used in general terms throughout the paper.

Four different methods were used to analyse the pigments in exoskeleton and muscle tissue:

1. In vivo OD (λ) (350–700 nm) on pieces of exoskeleton was obtained using a quartz cuvette in a split-beam mode spectrophotometer (Jenway 6715 UV/Vis. Spectrophotometer) according to Grzymski et al. (1997).
2. In vitro OD (λ) (350–700 nm) of raw pigment extracts from each specimen was obtained using a quartz cuvette in a double-beam spectrophotometer (Unicam UV 500 Thermospectronic spectrophotometer). Pigment raw extracts were made by grinding the exoskeleton and/or muscle tissue in a mortar in an organic solvent, methanol for *E. gryllus* and 7:3 methanol: acetone for *Anonyx* sp., respectively. The extracts were left in dark for 24 h in a −18°C freezer and then filtered through a 0.2-µm filter (13-mm syringe filter, Minisart RC 25) to avoid debris and corresponding light scattering.
3. High-performance liquid chromatography (HPLC) using a Hewlett-Packard 1100 series HPLC system equipped with a diode array detector (350–800 nm) obtaining OD (λ) according to the method of Rodrìguez et al. (2006) was used to isolate, identify and quantify the pigments present in the raw pigment extracts.
4. LC–MS (liquid chromatography–mass spectrometry) was performed according to the method of Stafsnes et al. (2010) on an Agilent time of flight (TOF) mass spectrometer equipped with an Agilent 1100 series HPLC system equipped with a diode array detector to determine the molecular weight of the carotenoids and thereby help in the identification process. Specifications were ±3 ppm error range and 10,000 in resolution.

The quantitative determination of the HPLC-isolated pigments in each colour stage was calculated using the response factor calculated from standards and the integrated area of the peaks at 440 nm in the chromatogram according to Eq. 1,

$$\frac{\text{ng}}{\mu\text{l}_{\text{extract}}} = \frac{(\text{area}_\lambda \times \text{Rsf}_\lambda)}{V_i} \quad (1)$$

where $\frac{\text{ng}}{\mu\text{l}_{\text{extract}}}$ is nanogram pigment per microlitre extract, 'area_λ' is the integrated area from each peak of the HPLC chromatogram, Rsf_λ is the response factor from the respective wavelength of the area and V_i is the injected volume in µl in the HPLC. The results from Eq. 1 were then calculated into 'µg/total extract' and divided by the wet weight to obtain µg/g wet weight. Response factors were calculated from standards; astaxanthin had an Rsf_{440}

at 0.222, cantaxanthin had an Rsf_{440} at 0.164, β,β-carotene had an Rsf_{440} at 0.084 and lutein had an Rsf_{440} at 0.161. The Rsf_{440} at 0.693 of diatoxanthin was calculated by the method of Jeffrey et al. (1997) in Eq. 2,

$$E^{1\%}_{\text{diatoxanthin}} = \frac{E^{1\%}_{\text{diadinoxanthin}} \times \text{MW}_{\text{diadinoxanthin}}}{\text{MW}_{\text{diatoxanthin}}}. \quad (2)$$

Results

Sample material

Despite similar set-up for the sampling, it was a marked difference in species composition between the 2 years. In 2007, all specimens caught (~500 individuals) consisted exclusively of *E. gryllus*. In 2008, however, all specimens (~400–500 individuals) belonged to *Anonyx* sp., with the exception of one single individual of *E. gryllus*. It was evident from the sampled material that some of the specimens of *E. gryllus* had undergone moulting inside the traps, since there were several empty exoskeletons among them. This was not detected for *Anonyx* sp.

Size

The results from the measurements of body length are presented in Table 1. A 2.2 fold increase in length in *E. gryllus* from colour stages I to IV was evident, while there was an increase in length from colour stages I to III in *Anonyx* sp. of 1.48. When performing an ANOVA on the body lengths of *E. gryllus*, there was a significant difference ($F(3, 8) = 81.12$, $P < 0.001$) between the body length of the different colour stages. A Tukey's HSD test showed that there was a significant difference ($P < 0.005$) between the mean body lengths of colour stages I and II, stages I and III, stages I and IV, stages II and III and stages II and IV, while there was no significant difference ($P > 0.05$) between colour stages III and IV. The ANOVA in *Anonyx* sp. showed a significant difference ($F(2, 6) = 28.60$, $P < 0.001$) between the body lengths of the different colour stages, while the Tukey's HSD test showed a significant difference ($P < 0.005$) between the mean body length of colour stages I and II and colour stages I and III, but no significant difference ($P > 0.05$) between colour stages II and III. The relationship between the in vitro OD (λ) and body length of *E. gryllus* ($R^2 = 0.8716$) and between the in vitro OD (λ) and body length of *Anonyx* sp. ($R^2 = 0.8239$) is displayed in Fig. 2.

Table 1 Mean body length (cm) ± SD of *E. gryllus* and *Anonyx* sp. in each colour stage

Colour stage	Body length (cm) ± SD	
	E. gryllus	*Anonyx* sp.
I	3.03 ± 0.20	2.77 ± 0.25
II	4.37 ± 0.41	3.83 ± 0.15
III	6.07 ± 0.25	4.10 ± 0.26
IV	6.67 ± 0.35	

$n = 3$ in all colour stages

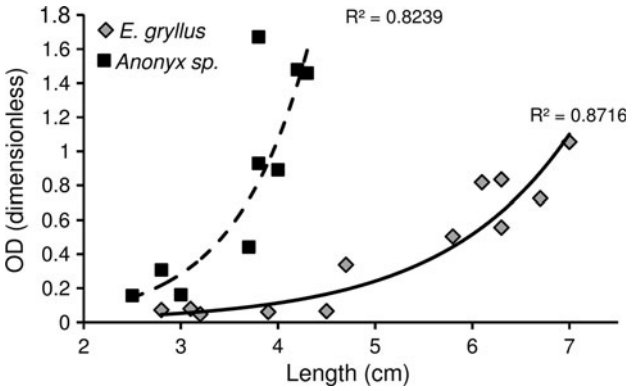

Fig. 2 Length versus OD with *R*-values in *E. gryllus* and *Anonyx* sp. indicating a exponential relationship between body length and colour intensity

In vivo OD (λ) characteristics

The measurements of in vivo OD (λ) in pieces of exoskeleton in *E. gryllus* indicated that there was at least one carotenoid present with spectral characteristics similar to that of astaxanthin and with a stronger spectral signature as a function of colour intensity (Fig. 3a). In *Anonyx* sp., the results were not as clear due to the fact that the pigments were located both in the exoskeleton (minor amounts) and in the muscle tissue within (major amounts). Only pieces of exoskeleton were measured with the "in vivo technique", because the tissue was too soft to use, but both tissue and exoskeleton were included in the extraction of pigments. However, although the results were variable, the spectral characteristic from the in vivo OD (λ) measurements did indicate carotenoids, with astaxanthin as the possible major pigment (Fig. 4a). Shifts in OD λ_{max} between the different colour stages in the in vivo measurements of exoskeleton were observed both in *E. gryllus* and in *Anonyx* sp. In *E. gryllus*, OD λ_{max} occurred at 487 nm in colour stage I and at 493 nm in colour stage IV. In *Anonyx* sp., OD λ_{max} was at 452 nm in colour stage I and at 455 nm in colour stage III.

In vitro OD (λ) characteristics

As in the in vivo measurements, the in vitro OD (λ) characteristic measurements of the pigment raw extracts in *E. gryllus* indicated that the main carotenoid was astaxanthin with a clear trend of increasing amounts of

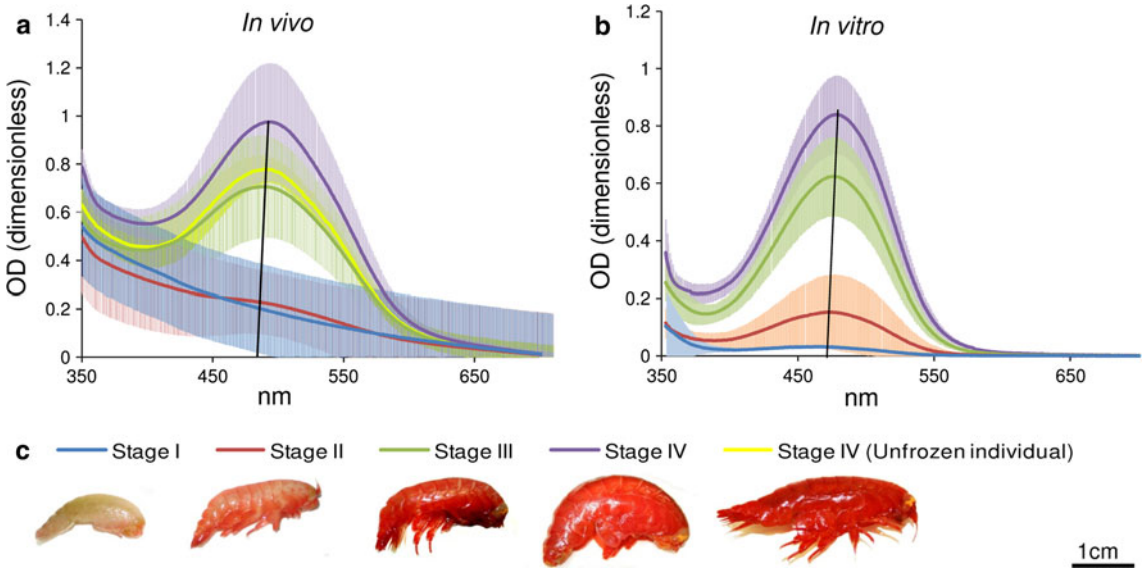

Fig. 3 **a** In vivo OD (±SD) of pieces of exoskeleton from each stage of colour in *E. gryllus*. OD (λ) indicates a stronger signature of astaxanthin as a function of colour intensity. Shifts in λ_{max} between the different colour stages are indicated by the *line*. **b** In vitro OD (±SD) of raw pigment extracts of the whole exoskeleton of *E. gryllus* in each stage of colour. OD (λ) indicates stronger signature of astaxanthin as a function of colour intensity. Shifts in λ_{max} between the different colour stages are indicated by the *line*. **c** Colour stages for *E. gryllus*

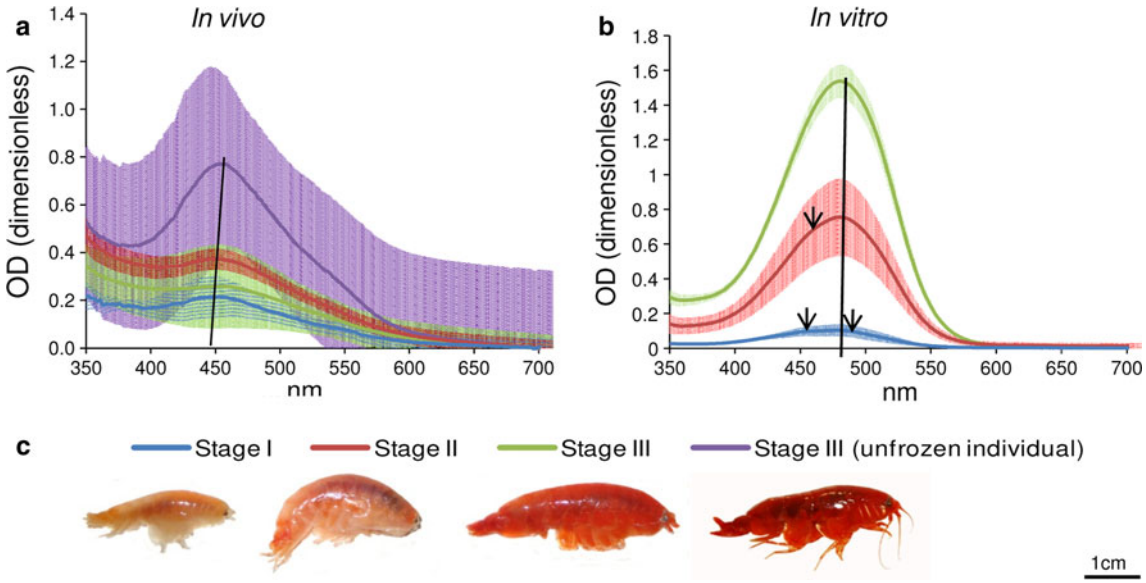

Fig. 4 **a** In vivo OD (±SD) of pieces of exoskeleton from each stage of colour in *Anonyx* sp. Shifts in λ_{max} between the different colour stages are indicated by the line. **b** In vitro OD (±SD) of raw pigment extracts of the whole exoskeleton and muscle tissue from *Anonyx* sp. in each stage of colour. OD (λ) indicates stronger signature of astaxanthin as a function of colour intensity. *Arrows* indicate shoulders. Shifts in λ_{max} between the different colour stages are indicated by the *line*. **c** Colour stages for *Anonyx* sp.

carotenoids as a function of colour intensity (Fig. 3b). In *Anonyx* sp., the OD (λ) characteristics also indicated an astaxanthin-dominated signature, but a change in shape of the OD (λ) between the different stages of colour indicated a mix of several pigments, which could occur in different amounts between the different stages of colour. It was also evident that the amount of carotenoids increased as a function of colour (Fig. 4b). As with the in vivo measurements, there were shifts in OD λ_{max} between the colour stages in the in vitro raw pigment extracts in *E. gryllus* and *Anonyx* sp. OD λ_{max} in *E. gryllus* at colour stage I was at 463 nm, while in colour stage IV OD λ_{max} occurred at 478 nm. In *Anonyx* sp., OD λ_{max} of colour stage I was at 479 nm, while OD λ_{max} in colour stage III was at 480 nm.

Spectral shifts in OD λ_{max} between average in vivo and in vitro material

When comparing the average of in vivo and in vitro OD (λ) from all colour stages, it was evident that there was a shift in λ_{max} from 486 nm in vivo to 476 nm in vitro in *E. gryllus* (Fig. 5a), which is a decrease in λ_{max} of 10 nm. On the other hand, there was a shift in λ_{max} from 453 nm in vivo to 480 nm in vitro in *Anonyx* sp. (Fig. 5b), which is an increase in λ_{max} of 27 nm. In both species, however, it was clear from plotting the difference spectra ($a\ (\lambda) - b\ (\lambda) = c\ (\lambda)$) that the in vivo materials have wider absorption window than in vitro.

Variability in OD in the samples from *E. gryllus* and *Anonyx* sp. is displayed in Table 2. The CV was usually not higher than 20–30% of mean value.

HPLC

The results from the pigment isolation by HPLC are compiled in Table 3, and an example of a HPLC chromatogram from colour stage II in *Anonyx* sp. is displayed in Fig. 6. The pigments were identified by comparisons with standard pigment-specific elution times and their corresponding OD (λ) characteristics. The major pigment fraction in both *E. gryllus* and *Anonyx* sp. was identified as astaxanthin, and it appeared predominant in all colour stages.

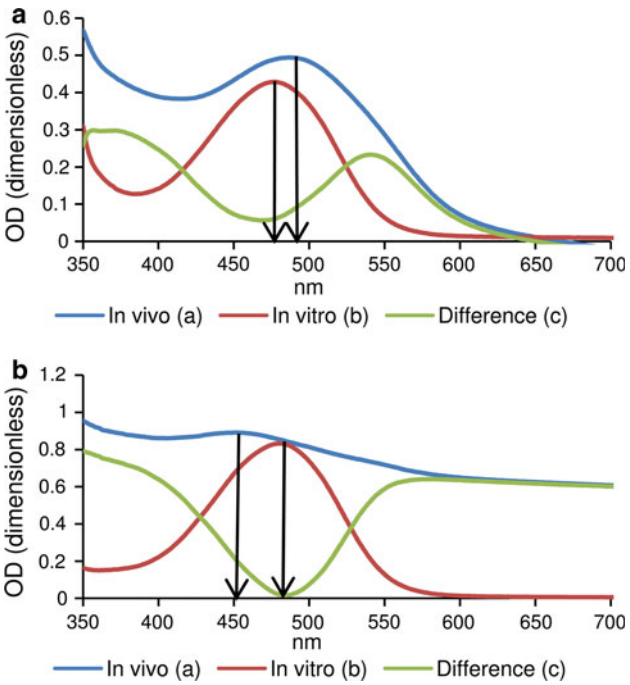

Fig. 5 **a** In vivo (*a*) and in vitro (*b*) OD (λ) and the difference between them (*c*) in *E. gryllus*. ($a\ (\lambda) - b\ (\lambda) = c\ (\lambda)$). **b** In vivo (*a*) and in vitro (*b*) OD (λ) and the difference between (*c*) in *Anonyx* sp. ($a\ (\lambda) - b\ (\lambda) = c\ (\lambda)$)

Table 2 OD λ_{max} and variation in *E. gryllus* and *Anonyx* sp. at different colour stages in both in vivo and in vitro measurements

	Colour stage	In vivo		In vitro	
		λ_{max}	CV (%)	λ_{max}	CV (%)
E. gryllus	I	487	20.71	463	20.77
	II	487	16.03	471	84.54
	III	487	20.29	476	21.85
	IV	493	18.02	478	15.71
Anonyx sp.	I	452	21.85	479	33.51
	II	453	13.43	480	29.41
	III	455	8.58	480	6.22

Eurythenes gryllus: Only astaxanthin was found in colour stage I of *E. gryllus*. In colour stage II, astaxanthin was still predominant, together with a lutein-like carotenoid. Colour stage III had, in addition to astaxanthin, a canthaxanthin-like carotenoid together with traces of three other carotenoids with OD maxima situated around 467 nm, 458–488 nm and 454–482 nm. However, the amounts of the unknown carotenoids were too small to identify them with any certainty [OD maxima lower than 5 mAU (milli-absorbance unit, absorbance = optical density (dimensionless))]. Colour stage IV had three forms of astaxanthin, a canthaxanthin-like carotenoid together with three unidentified carotenoids with OD maxima situated around 463 nm, 469 nm and 451–480 nm. The amount of astaxanthin I increased by a fourfold from colour stage I to stage IV in *E. gryllus*. There was a higher amount of astaxanthin I in colour stage III compared to stage IV.

Anonyx sp.: *Anonyx* sp. generally had a higher diversity of carotenoids than *E. gryllus*; in colour stage I, four forms of astaxanthin, two forms of diatoxanthin and β,β-carotene were present. There were two dominating forms of astaxanthin, one at 21 min (astaxanthin I) and one at 26.5 min (astaxanthin II, Fig. 6). The two forms were present in all the following colour stages. In colour stage II, astaxanthin I and II were present, together with two other forms of astaxanthin. In addition, there were three different forms of β,β-carotene, two forms of diatoxanthin and a cantaxanthin-like carotenoid, together with one unidentified carotenoid with OD max at 467 nm. Colour stage III had both astaxanthin I and II together with four other forms of astaxanthin, two forms of a cantaxanthin-like carotenoid, one β,β-carotene-like carotenoid and one unidentified carotenoid with OD maxima situated around 456–480 nm. Astaxanthin I increased 4.16 times from colour stage I to stage III, while astaxanthin II increased 4.23 times from colour stage I to stage III.

Table 3 Carotenoid composition in *E. gryllus* and *Anonyx* sp. μg/g wet weight and the corresponding ± SD are shown for each pigment stage (I–IV)

Carotenoids	Carotenoid composition (μg/g wet weight ± SD)						
	Eurythenes gryllus				*Anonyx* sp.		
	Colour stages				Colour stages		
	I	II	III	IV*	I	II	III
Astaxanthin I	9.40 ± 3.15	24.27 ± 21.34	72.20 ± 15.28	37.91 ± 11.20	5.85 ± 2.55	12.75 ± 8.51	24.35 ± 3.97
Astaxanthin II	–	–	–	–	7.16 ± 5.19	18.35 ± 4.66	30.03 ± 7.20
Astaxanthin-like	–	–	–	5.66 ± 1.63	2.77 ± 2.14	4.28 ± 1.93	9.90 ± 2.84
β,*β*-carotene	–	–	–	–	0.65 ± 0.10	0.30 ± 0.15	0.05 ± 0.01
Diatoxanthin	–	–	–	–	2.71 ± 0.97	2.88 ± 1.16	2.55 ± 0.16
Cantaxanthin-like	–	–	1.70[a]	2.09 ± 0.81	–	0.79 ± 0.11	1.17 ± 0.18
Lutein-like	–	1.50[a]	–	–	–	–	–
Unidentified compounds	–	–	Trace	Minor	–	Trace	Trace

$n = 3$ in each colour stage

* Sources of error: not completely in solution, H_2O in the extract (affects extraction efficiency), weight affected by high H_2O

[a] No SD because the carotenoid was only evident in one of the samples

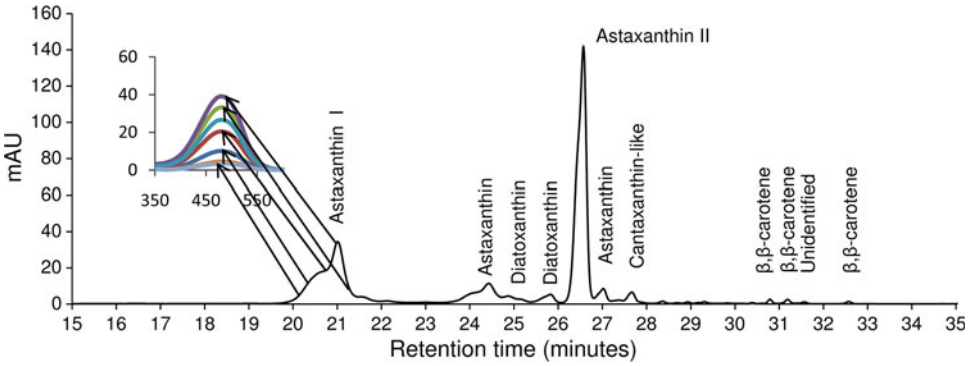

Fig. 6 HPLC chromatogram (mAU = milli OD at 440 nm) from colour stage II of *Anonyx* sp. Retention times are displayed in minutes. Different pigment peaks and shoulders denote a given pigment attached to unspecific macro-molecules that slightly affects the mobility (polarity), cf. astaxanthin I as an example

LC–MS

To verify pigment composition based on HPLC pigment-specific retention times and OD (λ) characteristics of standards, the corresponding pigment extracts were analysed in LC–MS (liquid chromatography-mass spectrometry) to obtain the molecular weights. Astaxanthin has according to the literature a molecular weight of 596.85 (Jeffrey et al. 1997). The standard astaxanthin in the LC–MS appeared at approximately 13.6 min, with a molecular weight at 596.39. The results from the LC–MS confirmed that the major pigment in both species was astaxanthin, with a molecular weight of 597.39 (since the LC–MS adds one proton, the molecular weight is about 1 higher than the average molecular weight). Astaxanthin occurred in all colour stages of both *E. gryllus* and *Anonyx* sp. at approximately 13.8 min. The other large elution peak of pigment in *Anonyx* sp. appeared at approximately 15.61 min and had the same OD (λ) characteristics as astaxanthin, but with a lower molecular weight of 577.52. In the contour plot from the LC–MS results, both *E.gryllus* and *Anonyx* sp. displayed substances that had some OD in the range from 250 to 350 nm, which is UV light, but these were not identifiable and not of carotenoid origin.

Discussion

Pigment composition

The results from the LC–MS chromatogram were compared with the results from the HPLC chromatogram to give a better indication of which pigments were present in the sample based on pigment-specific molecular weights. This confirmed that astaxanthin was the major carotenoid in all colour stages in both *E. gryllus* and *Anonyx* sp., which was indicated by the in vivo and in vitro OD measurements (Figs 3, 4, 5). The HPLC measurements indicated that there were several other carotenoids present both in colour stages II, III and IV of *E. gryllus* and in all colour

stages of *Anonyx* sp., although these were not detectable in the results from the LC–MS due to low amounts and less sensitivity in the LC–MS than in the HPLC. Still, by the use of retention time and spectral comparisons with standards, the HPLC results identified, in addition to the various forms of astaxanthin, a lutein-like and a canthaxanthin-like form in *E. gryllus* and *β*,*β*-carotene, diatoxanthin and a canthaxanthin-like form in *Anonyx* sp. In all of the results from the HPLC, the retention times of the samples were lower than the retention times of the standards. This was evident in astaxanthin, diatoxanthin, *β*,*β*-carotene and the canthaxanthin-like carotenoid and was probably due to unknown molecules which were bound to the carotenoids. There were also different retention times between the HPLC and the LC–MS due to different mobile phase composition.

Astaxanthin is often found in a variety of forms due to the two hydroxyl groups which can attach to a variety of ester linkages and thereby increase the number of possible forms (Bidigare et al. 1993), and this was evident both in the samples from *E. gryllus* and from *Anonyx* sp. While at most two forms of astaxanthin were found in *E. gryllus*, *Anonyx* sp. had a total of six forms in colour stage III. In the LC–MS results from *Anonyx* sp., the molecular weight of the second large top (at 15.58 min) of astaxanthin had a molecular weight of 577.52 with an λ_{max} at 482 nm. The low molecular weight could indicate that this form of astaxanthin was degraded.

When preparing the pigment extracts from the amphipods, it became clear that *E. gryllus* and *Anonyx* sp. contain their pigments in different locations. This difference in keeping the pigments either in the exoskeleton (*E. gryllus*) or in the muscle tissue (*Anonyx* sp.) indicates that even though the two species probably have very similar lifestyles, they still have evolved quite differently in terms of pigment-bonding sites. The high CV of the in vitro measurements in colour stage II of *E. gryllus* was probably due to a shortage of material, which led to one specimen being more pigmented than the two others during grouping of the specimens.

The higher amounts of astaxanthin in colour stage III compared to colour stage IV in *E. gryllus* did not correspond to the results obtained in the in vitro raw pigment extracts from the spectrophotometer, where the carotenoid content increased as a function of colour intensity. The calculations and measurements of pigments per wet weight may, however, been affected by two sources of error; first of all, while the wet weight of *E. gryllus* was measured on the exoskeleton only, this was not possible in *Anonyx* sp. due to problems with size of specimens and loss of material during weighing. This resulted in the whole body of *Anonyx* sp. being used in the weighing, which could be affected by factors like water and stomach content. Secondly, the weight of the exoskeleton in the specimens from colour stage IV in *E. gryllus* was almost twice that of the weights in colour stage III, possibly due to thickening of the exoskeleton with size and age or water contained in the pieces of exoskeleton. When calculating the $\mu g_{pigment}/\mu g_{wet\ weight}$, this would have influenced the calculations quite substantially.

Difficulties with the in vivo OD readings in the spectrophotometer, due to uneven pigmentation and differences in thickness of the exoskeleton pieces, was probably the reason for the variability in the measurements of in vivo pieces of exoskeleton in *Anonyx* sp., both regarding the higher amount of pigment in the unfrozen material compared to the frozen and that colour stage II exhibited more pigmentation than colour stage III.

An increase in pigmentation was evident when the in vitro OD was plotted as a function of length, in which both *E. gryllus* and *Anonyx* sp. had an exponential increase. There was also a significant difference in length between the colour stages with the least colouration (I and II) in both *E. gryllus* and *Anonyx* sp. This apparent correlation between colour intensity and size (length) could indicate that there is an increase in pigmentation with growth and increasing body size. In contrast, the difference in length between colour stages III and IV in *E. gryllus* and colour stages II and III in *Anonyx* sp. was not significant. This may indicate that growth ceases or slows down at a certain size, while the amount of pigmentation continues to increase.

Spectral shifts between colour stages

Spectral shifts in the OD λ_{max} between the different colour stages occurred in both in vivo and in vitro measurements in both species. In the in vivo measurements, *E. gryllus* displayed a shift of 6 nm between colour stages I and IV, while *Anonyx* had a difference of 3 nm between colour stages I and III. In the in vitro measurements, *E. gryllus* had 15 nm between colour stages I and IV, while only 1 nm separated colour stages I and III in *Anonyx* sp. These shifts were probably caused by changes in pigment composition between different colour stages and indicate both intra- and inter-specific differences.

Spectral shifts between average in vivo and in vitro measurements

The spectral shifts between the averaged in vivo and in vitro measurements were probably caused by pigments being bound to proteins, which were present in the in vivo material, but removed when the pigments were extracted. Interestingly, in *E. gryllus*, the shift went from long wavelengths in the in vivo measurements to shorter wavelengths in the in vitro measurements, while in *Anonyx*

sp. the shift went from shorter wavelengths in the in vivo measurements to longer wavelengths in the in vitro measurements, indicating interspecific differences.

Changes in pigment composition

The change in pigment diversity in the different stages of colour and body size in both *E. gryllus* and *Anonyx* sp. could be caused by uptake of carotenoids from different sources throughout the life of the amphipod. Ingram and Hessler (1983) and Smith and Baldwin (1984) found a relationship between increasing altitude above the seafloor and the average size of *E. gryllus*, which could indicate changes in diet throughout life. This could also help explain the advantage of the colour variation; younger individuals which spend most of their time close to the bottom would benefit from having a pale colour to blend in with the sediment, while larger individuals which spend more time higher up in the water column would be better camouflaged with a dark red colour. The diet may also be influenced by seasonal changes, since, as mentioned introductorily, most carotenoids in the deep-sea originate from phytoplankton found in surface waters (<100 m) and are transferred down the food web; seasonal changes in phytoplankton composition will subsequently influence carotenoid availability further down the food web. Baldwin and Smith (1987), on the other hand, suggested that carotenoid pigments accumulate during the intermoult period but are then lost again when moulting, leading to changes in pigment composition due to longer intermoult period in larger individuals (Ingram and Hessler 1987). This could again be influenced by the fact that organisms tend to live longer and grow to larger sizes in polar areas, a phenomenon often referred to as "Polar Gigantism" (Chapelle and Peck 1999). However, this would not be applicable to *Anonyx* sp., in which the majority of pigments are stored in the muscle tissue and are therefore not lost during moulting. Species-specific differences in metabolic pathways could be another explanation for changes in pigment composition between stages of body size, which causes one carotenoid to synthesize into another, as for example, β,β-carotene into astaxanthin, a well-known pathway in Crustacea (Tanaka et al. 1976; Berticat et al. 2000; Gaillard et al. 2004). Changes related to reproduction may also be likely, as carotenoids have been connected to several biological processes (Gilchrist and Lee 1972; Berticat et al. 2000; Liñán-Cabello et al. 2002) that could lead to changes in carotenoid composition between immature and mature females. Hargrave et al. (1994) suggested that due to increasing gonad volume taking up internal body volume during sexual maturation and breeding, feeding may actually be impossible in mature female amphipods. This was found to be the case in several *Anonyx* spp. by Sainte-Marie and Lamarche (1985) and would probably influence the carotenoid composition in the amphipods quite substantially.

It was evident from the presence of several empty exoskeletons that some specimens of *E. gryllus* had undergone moulting in the traps. This, however, was not found among the specimens of *Anonyx* sp. Being able to consume large amounts of food over a short timeframe would be advantageous for a deep-sea scavenger as food falls come rarely and unexpected. There are known adaptations in other species, where the amphipods have expandable stomachs and can swell up to three times their normal size (Shulenberger and Hessler 1974; Thurston 1979). *E. gryllus* is known to have a rigid exoskeleton (Dahl 1979), so the empty exoskeletons could therefore perhaps be explained by moulting, where an individual moults to get a softer and more expandable body, enabling larger amounts of food to be consumed. This would be, however, a quite risky strategy, as newly moulted individuals would be vulnerable to attacks from both intra- and inter-specific predators. A different explanation might be that the empty exoskeletons actually were eaten individuals, or that they had burst, which was the case in a study by Ingram and Hessler (1983).

Conclusion

The predominant pigment in all colour stages of both *E. gryllus* and *Anonyx* sp. was astaxanthin, which occurred in several forms, probably due to esterification, and which presence was confirmed by measurements of molecular weights from the LC–MS. In addition, by retention times and OD (λ) obtained by HPLC, several other carotenoids were identified; *E. gryllus* contained lutein-like and canthaxanthin-like carotenoids in addition to six unidentified carotenoids, while in *Anonyx* sp. β,β-carotene, diatoxanthin and canthaxanthin-like carotenoids, as well as two unidentified carotenoids, were found. An increasing amount and diversity of carotenoids as a function of colour intensity was evident in both species, and there was also a correlation between colour intensity and body size. There were indications that growth ceases or slows down at a certain size, while the amount of pigmentation continues to increase. Shifts in wavelength between the colour stages and shifts between the in vivo and in vitro average measurements indicated both intra- and inter-specific differences. Changes in pigment composition are probably affected by both diet and moulting/growth patterns and possibly also by other factors like metabolic pathways or reproduction. Due to the lack of knowledge about these species, future prospective would be to further elucidate their ecophysiology and distribution patterns, as well as general physiology and ecology.

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