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Short communication

Limiting immunopathology: Interaction between carotenoids and enzymatic antioxidant defences

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ABSTRACT

The release of reactive oxygen and nitrogen species (ROS and RNS) during the inflammatory response generates damages to host tissues, referred to as immunopathology, and is an important factor in ecological immunology. The integrated antioxidant system, comprising endogenous antioxidant enzymes (e.g. superoxide dismutase SOD, and catalase CAT) and dietary antioxidants (e.g. carotenoids), helps to cope with immune-mediated oxidative stress. Crustaceans store large amounts of dietary carotenoids for yet unclear reasons. While being immunostimulants and antioxidants, the interaction of these pigments with antioxidant enzymes remains unclear. Here, we tested the interaction between dietary supplementation with carotenoids and immune challenge on immune defences and the activity of the antioxidant enzymes SOD and CAT, in the amphipod crustacean *Gammarus pulex*. Dietary supplementation increased the concentrations of circulating carotenoids and haemocytes in the haemolymph, while the immune response induced the consumption of circulating carotenoids and a drop of haemocyte density. Interestingly, supplemented gammarids exhibited down-regulated SOD activity but high CAT activity compared to control ones. Our study reveals specific interactions of dietary carotenoids with endogenous antioxidant enzymes, and further underlines the potential importance of carotenoids in the evolution of immunity and/or of antioxidant mechanisms in crustaceans.

1. Introduction

During the inflammatory response, the release of highly reactive oxygen and nitrogen species (ROS and RNS) generates damages to host tissues, referred to as immunopathology, and contributes to immune system costs uncovered in evolutionary studies (Pursall and Rolff, 2011). Among the protective processes limiting inflammatory costs, hosts evolved the antioxidant defence system controlling the amount of circulating cytotoxic ROS and RNS. This system comprises key endogenous antioxidant enzymes, including the superoxide dismutase (SOD) and catalase (CAT), which are successively involved in the detoxification of superoxide radicals, and the action of dietary antioxidants such as carotenoids (Felton and Summers, 1995). While immunostimulants (e.g. Park et al., 2010), carotenoids also scavenge free radicals (e.g. ROS and RNS), which may limit the costs of inflammation (e.g. Walrand et al., 2005).

Despite their involvement in the antioxidant defence system, the interaction of dietary carotenoids with the key endogenous antioxidant enzymes SOD and CAT remains unclear. On the one hand, carotenoids may take over the action of the antioxidant enzymes (Chien et al., 2003; Wang et al., 2006), reducing their relative activity and therefore their maintenance costs. On the other hand, in addition of being free radical scavengers, carotenoids were suggested to stimulate the activity of these antioxidant enzymes (Kim et al., 2005; Lee et al., 2011), which would enhance detoxification efficacy upon immune activity. In both cases, carotenoids may improve the detoxification of free radicals, alleviating the costs associated with oxidative stress. This might be particularly relevant for animals storing large amounts of carotenoids in their tissues.

Crustaceans store naturally large amounts of dietary carotenoids, mainly astaxanthin and lutein, in their tissues (Dembitsky and Rezanka, 1996; Gaillard et al., 2004) for yet unclear reasons. In the amphipod, *Gammarus pulex*, the amount of carotenoids in the haemolymph varies among natural populations and correlates positively with immune defences (Cornet et al., 2007). Crustacean immunity is innate and relies on the response of immune cells and rapidly activated enzyme cascades such as the prophenoloxidase (proPO) that are at the core of the inflammatory response (Nappi and Ottaviani, 2000; Söderhäll and Cerenius, 1998). Interestingly, the experimental supplementation of the food of *G. pulex* with carotenoids broadly stimulated innate immune defences without increasing the inflammation cost (Babin et al., 2010). Beneficial effects of dietary carotenoids combined to the high capacity of crustaceans to store these pigments suggest that the evolution of crustacean immunity may occur in a particular context. By

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reducing inflammation-associated costs, carotenoids may alleviate part of this constraint on the evolution of the immune function (Babin et al., 2010). To better understand the potential importance of carotenoids in the evolution of immunity and/or of antioxidant mechanisms in crustaceans, we aimed to test whether the action of these pigments stored by crustaceans is additive or synergistic with that of endogenous antioxidant enzymes to reduce immunopathology.

To this purpose, we tested the influence of food supplementation with carotenoids on important cellular and humoral components of the immune system of *G. pulex* (density of haemocytes, activity of the proPO system) and on the activity of the key endogenous antioxidant enzymes SOD and CAT, after a bacterial immune challenge.

2. Methods

2.1. Experimental design and measures

One thousand male gammarids were collected in March 2011 in the river Suzon (Val-Suzon, eastern France), and maintained individually in glass vials with 20 mL of oxygenated dechlorinated UV-treated tap water under standard laboratory conditions (15 °C ± 1 °C, 12:12 light:dark cycle). Immediately after field collection, gammarids were starved for 3 days before exposure to the food treatment to exacerbate hunger (Babin et al., 2010). For the food treatment, 500 gammarids were supplied with food supplemented with carotenoids (carotenoid food treatment) whereas the other 500 were offered the same food basis as in the carotenoid food treatment but without carotenoids (control food treatment). The food recipe is identical to the one used by Babin et al. (2010). The food treatment lasted 17 days during which gammarids were provided daily with food ad libitum and the remains from the previous provision were removed to keep the containers clean. Survival was checked daily and water was changed once a week to provide sufficient oxygen to the gammarids.

After the food treatment, 150 supplemented and 150 non-supplemented gammarids were randomly assigned to one of the three immune treatments: 'naive', 'PBS' and 'bacteria' to test interactions between immune challenge and carotenoid supplementation on carotenoid concentration, haemocyte density, activities of the proPO system in their haemolymph, and activity of the SOD and CAT enzymes of their antioxidant system from their entire body.

The 'naive' group was unchallenged. The 'PBS' group received a single injection of 0.5 µL of sterile phosphate buffer saline (PBS: 8.74 g NaCl; 1.78 g Na_2HPO_4, $2H_2O$; 1000 mL of distilled water; pH 6.5). The 'bacteria' group received an 8×10^5 cells.mL^{-1} dose of heat-killed *Escherichia coli* (CIP 103410, Institut Pasteur, Paris, France; inactivated 5 min at 100 °C) in 0.5 µL of PBS. While non-pathogenic, heat killed-bacteria stimulate the innate immune response, leading to the production of cytotoxic ROS and RNS in invertebrates (Nappi and Ottaviani, 2000), and promote immunopathology (Sadd and Siva-Jothy, 2006). Bacterial growth and injection procedures were done as described in Cornet et al. (2009). After injection, gammarids were maintained individually under standard conditions without food for 15 hours before haemolymph sampling. This is the time required for the immune response of *G. pulex* to complete bacterial clearance and restore homeostasis (Moret, personal communication).

Haemolymph was collected as described in Cornet et al. (2007). Each gammarid provided up to 3 µL of haemolymph, immediately diluted in 20 µL of ice-cold PBS, vortexed and split into a 10 µL sample for carotenoid dosage and a 13 µL sample for immune parameters, either haemocyte density or activities of the proPO system. Samples allocated to measure carotenoid concentration and activities of the proPO enzyme, as well as individuals, were frozen in liquid nitrogen and stored at –80 °C until later examination. Samples allocated to measure density of haemocytes were immediately treated using a Neubauer-improved haemocytometer under a microscope (magnification ×400).

Carotenoids were extracted and quantified via a colorimetric assay following the method of Cornet et al. (2007). Values were corrected to obtain concentrations for 1 µL of pure haemolymph. The activities of naturally activated PO (PO activity) and that of the pro-enzymes (which give the total PO activity when added together) were measured using a spectrophotometric assay following Cornet et al. (2009). Enzyme activity was measured as the slope (V_{max} value in milli-absorbance.min^{-1}) of the reaction curve using the software SOFT-Max®Pro 4.0 (Molecular Devices), and reported as the activity of 1 µL of pure haemolymph.

SOD and CAT enzyme activities were scored spectrophotometrically following the protocol of De Block and Stoks (2008). SOD activity was quantified based on the protocol from the SOD assay kit from Cayman Chemical (Ann Arbor, MI, USA), which measures the formation of formazan dye upon reduction of tetrazolium salt with superoxide anions. SOD activity was expressed in units (U) of SOD per milligram of gammarid fresh mass (U/mg FM). One absorbance unit of SOD corresponds to the amount of enzyme needed to inhibit the colorimetric reaction by 50%. CAT activity was quantified following a protocol adapted from Aebi (1984). We measured the removal of the H_2O_2 as the reduction in absorbance at 240 nm within 3 min at 25 °C. CAT activity was expressed as the decline in absorbance units (U) per min and per mg of gammarid fresh mass (U/min/mg FM) (see details in Supplementary material). Because we aimed to examine variation at the level of the whole organism and compare individuals, the measurements discussed earlier are expressed per unit of volume of haemolymph (e.g. concentration of carotenoids, density of haemocytes and activity of the proPO system) or unit of fresh mass (e.g. activity of the SOD and the CAT enzymes).

2.2. Statistics

Gammarid survival during the first 17 days of food treatment was analysed using a chi-square test. The effect of the immune challenge on carotenoid concentration, PO activity, total PO activity, density of haemocytes, SOD activity and CAT activity in interaction with the food treatment, was analysed using a linear model for each dependent variable including gammarid fresh mass as covariate, food treatment and immune treatment as fixed factors, and their two-way interactions. Non-significant interaction terms were removed from the models. Data of the six dependent variables were square root transformed to homogenise the variance. All data were analysed using SPSS 19 for Macintosh.

3. Results/discussion

Over the 17 days of food treatment, dietary supplementation with carotenoids significantly improved survival (75.2% versus 60.8%, $\chi^2_{[1]} = 23.17$, $P < 0.001$). Supplemented gammarids had higher carotenoid concentrations in their haemolymph (Fig. 1A; Table 1) within the natural range (up to 1840 ng/µL; Cornet et al., 2007) and exhibited stronger reddish coloration (Supplementary Fig. S1). The immune treatment affected carotenoid concentration in interaction with the food treatment (Table 1). Whereas the carotenoid concentration of supplemented gammarids remained high across immune treatments, the immune challenge with heat-killed bacteria consumed a significant amount of these circulating pigments in non-supplemented ones (Fig. 1A). This clearly illustrates the physiological involvement of carotenoids in the immune response, probably by scavenging the excess of free radicals produced by immune activity (Walrand et al., 2005). Saturation of the haemolymph with carotenoids in supplemented gammarids may