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Copepods' true colors: astaxanthin pigmentation as an indicator of fitness

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Abstract

Pigmentation is often overlooked in zooplankton, since these organisms are mostly colorless to fit the translucid water medium. However, one of the dominant zooplankton taxa in aquatic ecosystems—copepods—often show a bright red-orange or blue coloration owing to the accumulation of carotenoid pigments in some parts of their bodies. Even though there are many functional traits describing copepod's performance (e.g., size, feeding, and reproductive modes), it is surprising that the role of such a simple and visible trait as coloration has not been studied in a coherent manner yet. Here, by reviewing 95 studies, we demonstrate that carotenoid-based pigmentation (mainly caused by astaxanthin molecules) is a widespread functional trait in freshwater and marine copepods. We propose a way to disentangle the complex and thus intriguing patterns of pigment expression along latitudinal and altitudinal gradients, addressing its relationship to diet quality and quantity, temperature, ultraviolet radiation stress, predation pressure, lipid metabolism, and reproduction. We show that large-scale variations in pigmentation are difficult to tackle because of the fundamental plasticity of this trait at short time scales (i.e., hours, days), and the most recent information about carotenoid bioconversion are addressed (genes and enzyme identification, and influence of microbiota). From this literature review, we hypothesize that pigments play a

"Swiss-army knife" role for copepod's fitness, useful in various ecosystem conditions owing to the strong antioxidant power and the finely-tuned metabolism of astaxanthin. With larger antioxidant capacities (survival), higher metabolisms (growth), and more offspring in better condition (reproduction), red morphs appear more successful than their uncolored siblings. Also, the potential camouflage strategies allowed by red and blue pigmentation are discussed. We finally formulate new directions and future research fields from molecular to ecosystem scales. Routine quantifications of copepod's pigmentation through trait-based approaches could be useful (1) to obtain an accurate copepod fitness indicator and (2) to better estimate the transfer of antioxidant to higher trophic levels in ecosystems, including humans.

INTRODUCTION

Color is a morphological property that can be assessed at the individual level and that impacts survival, growth, and reproduction of organisms; as such it corresponds to the definition of a functional trait (Violle et al., 2007). Carotenoid-based colors are caused by the presence of conjugated double-bond molecules able to absorb light at wavelengths of 400–500 nm in the visible region of the spectrum (de Carvalho & Caramujo, 2017). These pigments are ubiquitous in animals and are known to influence individual fitness thanks to two distinct properties (Weaver, Wang, et al., 2018). First, they are responsible for behavioral responses because strong pigmentation influences prey/predator interactions (e.g., red prey can be more targeted or camouflaged; Hansson, 2004; Johnsen, 2014) and sexual selection by conveying a true indicator of individual health condition ("honest signal of fitness"; McGraw, 2005; Negro et al., 1998; Seehausen, 1997; Torres & Velando, 2005). Second, carotenoid pigments are electron acceptors with higher affinities than other cellular components, providing protection against oxidative damage on lipids, proteins, and DNA. Numerous studies demonstrate that the accumulation of carotenoids improves animal health: it reduces disease occurrence in fish (Nakano & Wiegertjes, 2020; Pettersson & Lignell, 1999; Pickova et al., 1998) and can regulate immune functions and cancer proliferation in mammals (Chew & Park, 2004), including humans (Donoso et al., 2021). The study of carotenoid-based color in animals is thus at the interface of physiology, ecology, and behavioral studies and is well amenable to a functional trait-based approach (Martini et al., 2021).

Copepods are small planktonic crustaceans inhabiting oceans, rivers, lakes, and ponds, and are probably the most abundant metazoans on our planet (Humes, 1994).

They represent a crucial link between micro-planktonic prey and higher consumers in aquatic food webs (Cavallo & Peck, 2020). Moreover, they contribute to the biological carbon pump in the ocean by the production of sinking fecal pellets and by their vertical migrations (Jónasdóttir et al., 2015; Turner, 2015). Their ecological importance is driven by several key functional traits, including their size distribution, feeding modes, and dormancy strategies (Benedetti et al., 2016, 2022; Brun et al., 2016; Kiørboe et al., 2015). Color, in contrast, is a trait often overlooked in zooplankton organisms (including copepods), probably because it is assumed that transparency is the main strategy to hide in the water medium (Johnsen, 2001). From a practical point of view, this general lack of interest might be caused by the typical preservation of plankton samples in fixatives (e.g., ethanol, formaldehyde) that lead to the leaking or oxidation of pigments, making color information unreliable. Nevertheless, red pigmentation in copepods was described in two species, *Diaptomus bacillifer* and *Acanthodiaptomus denticornis* as early as the 19th century in lakes of the French Alps (Blanchard, 1890). Similar observations were later made in northern American and Swedish lakes (Brehm, 1938; Byron, 1982; Hairston, 1976, 1979; Hansson, 2004). From early on, red color was attributed to carotenoids. As in most crustaceans, astaxanthin and canthaxanthin are the principal carotenoid types found in copepods (Czeczuga & Czerpak, 1966; de Carvalho & Caramujo, 2017; Fisher et al., 1964; Matsuno, 2001). Pigmented copepods are found in both marine and freshwater environments, particularly in polar and subpolar regions (Hylander et al., 2015), but also in subtropical systems (Lee et al., 2019). The red copepods from the North Atlantic (*Calanus* spp.) are valued in marine fisheries and aquaculture where they are

sometimes nicknamed "red feed" (Pedersen et al., 2014). Their carotenoid-rich oil is now sold for human health as an antioxidant, anticholesterolemic, and anti-inflammatory diet supplement (Gasmi et al., 2020). In equatorial regions, redness is much scarcer, but a complex of carotenoids and proteins can lead to a blue coloration, which is very common in neustonic copepods (Herring, 1965; Rahlff et al., 2018).

It is surprisingly complex to disentangle the physiological and ecological causes of the copepods' pigmentation. The main historical hypotheses tested in the literature (Table 1) concern (1) the photoprotective role of astaxanthin against solar radiation (Hairston, 1976), (2) the potential for heat absorption conferred by pigments that could lead to higher metabolic rates in individuals (metabolic stimulation hypothesis; Byron, 1982),

TABLE 1 Summary of historical and new hypotheses that have emerged to explain carotenoids accumulation in copepods.

Hypothesis	Description	Confidence[a]	References
H1: Photoprotective role hypothesis	Carotenoid pigments protect the whole individual against reactive oxygen species (ROS) produced by short light radiation	+++	Hairston (1976)
	Under light stress, a trade-off can occur if visual predators are present	++	Hansson (2004); Hylander et al. (2009)
H2: Metabolic stimulation hypothesis	Carotenoid pigments confer a potential for heat absorption leading to higher metabolic rates for redder individuals	(−)	Byron (1982)
H3: Food reserve hypothesis	Association of carotenoids with lipid reserves, both would be consumed as food in the future	(?)	Ringelberg and Hallegraeff (1976)
H4: Lipid protection and metabolism hypothesis	Carotenoids are synthetized and stocked with lipids as a protection against ROS mostly produced by oxygenated active metabolism, and by other external stressors	++	Foss et al. (1987); Grosbois and Rautio (2018); Schneider et al. (2016)
H5: Egg and nauplii protection hypothesis	Accumulation of carotenoids in eggs, often transferred from lipid reserves, as a protection against ROS produced by various sources (examples: by active metabolism during rapid growth or induced in buoyant eggs by light radiation in surface waters)	+++	Lotocka (2004); Schneider et al. (2016)
	Astaxanthin forms (free, esters, and caroteno-proteins) may thus change according to ontogeny	++	Lotocka (2004)
H6: Adaptive camouflage hypothesis	Red carotenoid coloration protects copepods by making them cryptic in deeper and darker waters, especially under the green-blue searchlights of predators	+	Johnsen (2005); Vestheim and Kaartvedt (2006)
	Coupling with proteins induce color changes (blue color) in relation with depth or daily cycles as a camouflage strategy	(?)	This article
H7: Mating criteria hypothesis	Accumulation of red carotenoids in females to be assessed by males ("honest signal") during mating	(−)	Powers et al. (2019)
H8: Antioxidant "Swiss army knife" hypothesis	Astaxanthin provides protection against all ROS, especially from endogenous sources, and can improve growth, reproduction and survival at various moment of copepods' life cycle	+++	Gorokhova et al. (2013); Lotocka (2004); Schneider et al. (2016); This article
	Astaxanthin bioconversion and thus color and its intensity, are very plastic and finely regulated (enzyme activities, gene transcription) according to the internal and external conditions (visibility condition, living depth, reproductive status, lipid storage, etc.)	+++	Gorokhova et al. (2013); Hylander et al. (2009); Johnsen (2005); Vestheim and Kaartvedt (2006); This article

[a]Confidence levels have been estimated from this literature review: (−): hypothesis theoretically or experimentally refuted, (?): hypothesis still to be tested, +: hypothesis with low level of confidence, ++: hypothesis with medium level of confidence, +++: hypothesis with high level of confidence.

and (3) the use of astaxanthin esters as food reserves in association to lipid storage (Ringelberg & Hallegraeff, 1976). The well-documented photoprotection hypothesis (Table 1, H1) has been shown to be related to the buffering of reactive oxygen species (ROS) that are produced by the activity of high-energy solar radiation. Regarding the metabolic stimulation hypothesis (Table 1, H2), it was argued that heat absorption by pigments in such small animals would lead to an insignificant temperature gain (Hairston, 1981). A higher metabolism in red individuals could be achieved not by heat increase, but rather by a better ROS buffering, thanks, again, to the protective action of carotenoids (Gorokhova et al., 2013; Hairston, 1976). Finally, the food reserve hypothesis (Table 1, H3) still lacks experimental confirmation, while actually raising another question: is there any reason why lipid storage is associated with red pigments? Thus, a more recent hypothesis concerns the specific protection of lipids (Table 1, H4) against ROS induced by all types of oxidative stressors, not only by light (Schneider et al., 2016).

In this review, we present the current knowledge about the occurrence of carotenoid-based pigmentation in copepods, the biochemical processes leading to carotenoid accumulation, and its diverse physiological, biological, and ecological consequences. In the light of the powerful capacity of the carotenoid molecules to intercept ROS, we demonstrate that all hypotheses could be further discussed and argued (Table 1). We also comment on color changes in relation to vertical migration and predation pressure. We will not discuss black pigmentation, sometimes described in the copepods *Candacia* sp. (Culverhouse et al., 2006; Purcell, 1983; Wilson, 1950), because this color is almost undocumented and has not been associated to carotenoid pigments. Color variations related to the structure of the exoskeleton rather than to pigment molecules, that is, iridescent Sapphirinid copepods (Kimura et al., 2020), are neither part of this review. We addressed three research questions: (1) To what extent is carotenoid-based color a common functional trait in copepods? (2) Which abiotic and biotic drivers influence carotenoid accumulation in freshwater and marine copepods? (3) What ecological advantages could red and blue pigments bring for individual fitness? To do so, we have retrieved information from 95 studies that contained the words "copepod" associated with "carotenoid*," "astaxanthin," or "redness" anywhere in the text body. Carotenoid concentration ranges were identified according to ecosystem (freshwater vs. marine), location (latitude, longitude, and altitude), taxonomy, and a few other contextual variables (Data1 and Data2). Another dataset was constructed to classify forcing variables (e.g., temperature, ultraviolet radiation, or diet composition) according to their impact on carotenoid concentrations (e.g., positive, neutral, and negative; Data3).

Data obtained from literature search was finally organized to list the effects of red pigmentation on fitness-related variables in copepods, such as their survival rates, swimming speed, RNA:DNA metabolic index, and 21 other indices assessing survival, growth, or reproduction (Data4). Datasets 1–4 and codes are available at https://doi.org/10.5281/zenodo.7671742, and easily visualized in a html file associated with the Rmarkdown code (Vilgrain, 2023, RMarkdown: Allaire et al., 2021; R Core Team, 2021; R version 4.0.3). In the metadata found at https://doi.org/10.5281/zenodo.7671742, we provided further details on how quantitative and qualitative information was chosen from the literature and reported within the datasets.

There are many possible reasons for copepods to exhibit red-orange or blue pigmentation (Table 1), and this property strongly varies in time and space. Our goal is to review and summarize the scattered knowledge about carotenoid accumulation in copepods. From this synthesis effort, we aim to unravel the copepod color puzzle and try to formulate new directions and future research fields broaden by this important trait (Table 2). Ubiquitous and accurate quantification of pigmentation in copepods could then lead to new insights in pelagic ecosystems functioning and to a better monitoring of aquatic ecosystem health.

CAROTENOID RED AND BLUE PIGMENTATION IN COPEPODS

A shared and conserved functional trait

From 95 articles containing the keywords "*copepods*" and "*carotenoid*," "*astaxanthin*," or "*redness*," it appears that carotenoid-based color is a very common functional trait in copepods, as it was observed in at least 154 distinct locations in the world (Figure 1). About 170 freshwater and marine copepod species from 65 genera are known to accumulate these pigments, and among these, the marine *Calanus* spp. and the freshwater *Diaptomus* spp. are the most studied (more than 30 reported observations each, see Data1). Of the 95 studies, 74% have quantified carotenoids, however, using various methods and units (Data1). Note that copepods have to be frozen (e.g., for chemical analyses) or taken in photos soon after sampling (within a few hours) to study their pigmentation. For all the following quantitative analyses, we used carotenoid concentrations measured by high-performance liquid chromatography (HPLC) and expressed in micrograms per milligram of dry mass (usually written dry weight, but noted dry mass here to follow the conventions of the SI system). It was the most common method and unit in the literature (202 over 286 measurements for

T A B L E 2 Suggestions for potential research fields associated with astaxanthin pigmentation in copepods.

Scale	Study suggestion	Existing literature
Molecular	Molecular cloning of enzyme-coding genes or mutant analysis, to identify astaxanthin biosynthesis enzymes potentially linked to mitochondrial functioning	Mojib et al. (2014); Powers and Hill (2021); Powers et al. (2022)
	Performing transcriptomics approaches to characterize fine-tuned controls on pigment synthesis in response to light or predators induced stress	Gorokhova et al. (2013); Hylander et al. (2009)
	Investigating the molecular link between lipid and astaxanthin synthesis	Grosbois and Rautio (2018); Schneider et al. (2016)
	Exploring microbiota composition in relation with pigmentation	Chae et al. (2021)
	Understanding molecular controls of blue to red color change according to time or between body structures (i.e., eggs)	Herring (1965); Mojib et al. (2014); Vestheim et al. (2005)
	Investigating the temperature dependence of astaxanthin stability (in its various forms, including caroteno-proteins)	Pedersen et al. (2014); Rahlff et al. (2018); Schneider et al. (2016)
Individual/Population	Determining concentrations and proportions of astaxanthin forms (free, esters, caroteno-protein) with ontogenic development	Cui et al. (2021); Lotocka (2004); Schneider et al. (2016)
	Assessing eggs hatching success and nauplii survival according to astaxanthin content	Hylander et al. (2009); Powers and Hill (2021)
	Investigating pigmentation variations according to living depth and/or to vertical migration	Hays et al. (1994); Vestheim et al. (2005)
	Testing sexual selection by blue pigmentation for neustonic copepods	Cohen and Forward (2002)
	Developing routine measures of pigmentation as one fitness index (e.g., using individual images or remote sensing)	Basedow et al. (2019); Brüsin et al. (2016); Lindeque et al. (2022)
Ecosystem	Investigating copepods' astaxanthin content according to latitude at global scale	Hansson et al. (2007); Hylander et al. (2009)
	Testing red color as a camouflage strategy at depth	Johnsen (2001, 2005); Vestheim and Kaartvedt (2006)
	Exploring the impact of anthropogenic pressures (pollutants, eutrophication) on astaxanthin production and copepod fitness	Caramujo et al. (2012); Holeton et al. (2009); Medina et al. (2008)
	Exploring the link between copepod pigmentation and fish population success	Azani and Rasdi (2021); Hynes et al. (2009)

which latitude, longitude, altitude, species, life stages, and astaxanthin concentrations were provided). Because analytical machines are not capable of resolving too-small amounts of molecules, a measurement is here a HPLC quantification of pigments performed on a sample that contains multiple individuals (e.g., more than 10), then normalized by the mass of the sample. Whenever possible, we have converted some carotenoid quantifications expressed in other units, insofar they were extracted by chemical methods for monospecific samples of late copepodites or adults (details in Appendix S1: Table S1). Finally, 211 values in $\mu g\ mg\ DM^{-1}$ were used for quantitative analyses in this article. Globally, carotenoid concentrations range from 0.00014 to 17.2 $\mu g\ mg\ DM^{-1}$ with an average of 1.22 $\mu g\ mg\ DM^{-1}$. This data also allowed us to confirm previous findings (e.g., Hylander et al., 2014) that freshwater species concentrate more pigments than marine species, with a median of 2.243 $\mu g\ mg\ DM^{-1}$ ($n = 66$) versus 0.137 $\mu g\ mg\ DM^{-1}$ ($n = 145$), respectively (Figure 1 and Appendix S1: Figure S1 for details by copepod genus). To statistically compare carotenoid concentrations between marine and freshwater ecosystems, we performed a Wilcoxon rank sum test because data on marine carotenoid concentrations were still not normally distributed after logarithmic transformation. This test was highly significant ($p < 0.001$) and highlighted the
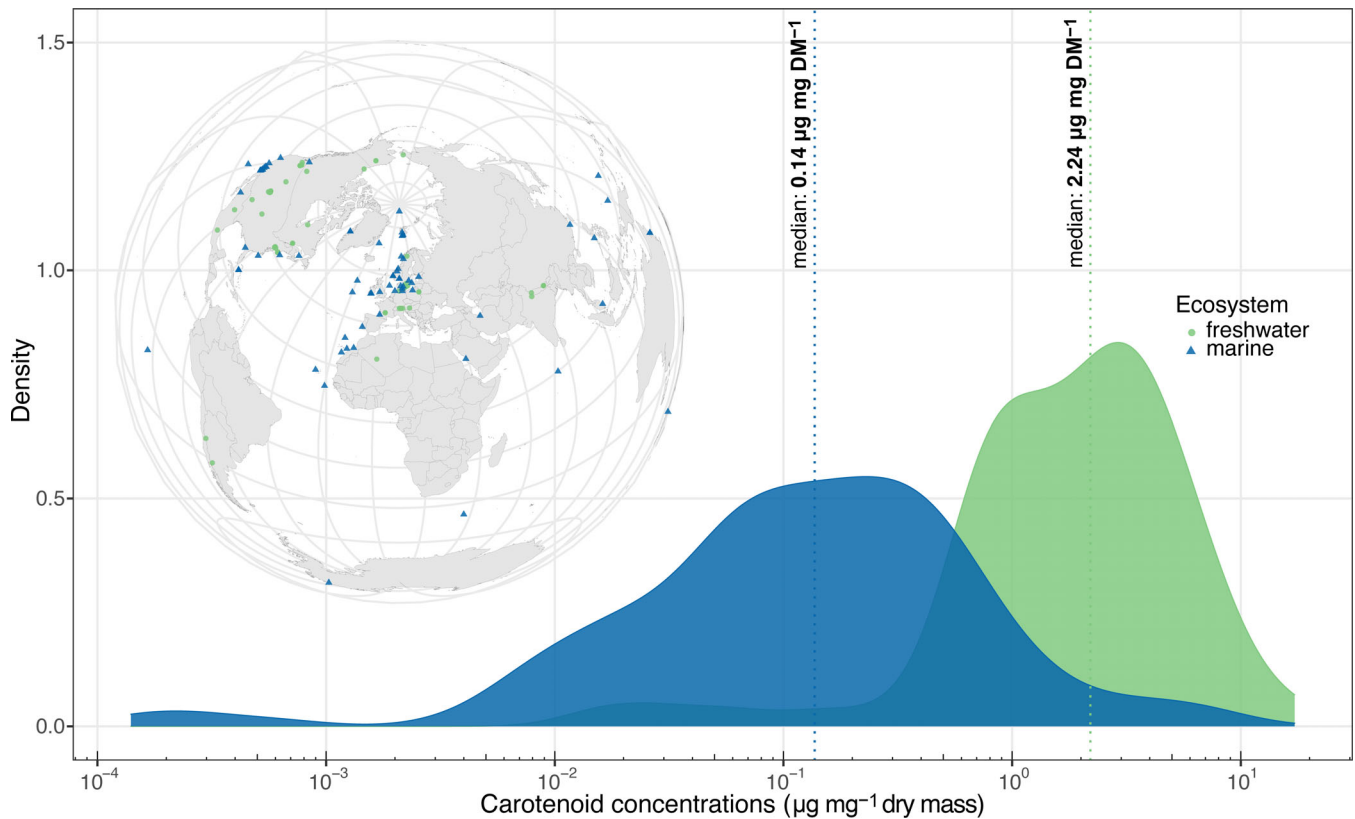
FIGURE 1   Carotenoid concentrations in freshwater and marine copepods across the world. Observations of pigmented copepod samples are available at 154 locations globally (inset map) from 95 studies published since 1949. Freshwater is in green and marine in blue. We used 211 pigments measurements (in micrograms per milligram dry mass) to plot density distributions of carotenoid concentrations with a log-transformed x-axis. The median copepod carotenoid content is higher in freshwater than in marine ecosystems (Wilcoxon rank sum test, $p < 0.001$).
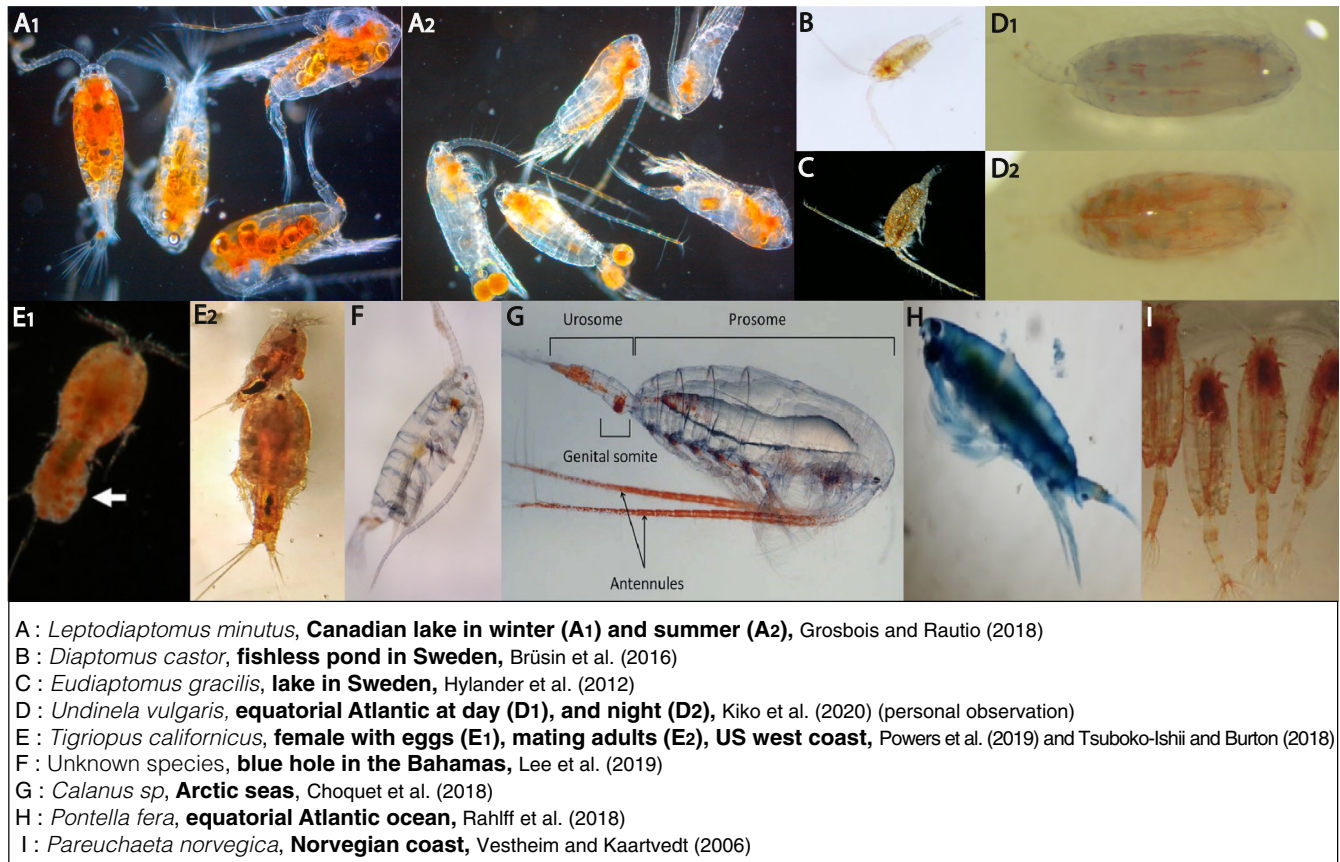
strong difference in carotenoids accumulation by copepods between the two aquatic ecosystems.

Localization, molecular forms, and metabolism of carotenoid pigments

Carotenoids induce an orange to red—and sometimes even blue—coloration in copepods of many different species. These species inhabit environments characterized by various abiotic and biotic pressures, which is an indication that the utilization of carotenoids presents diverse and potentially positive roles in copepod biology. Disparities in carotenoid localization and forms result in different visual signatures, even for the same pigment quantity (Herring, 1972) (Figure 2). Indeed, coloration can be uniformly spread throughout the copepod's body, or clustered in some specific structures such as lipid droplets and lipid sacs, eggs, antennules, or genital somites (Figure 2). Although a few authors mentioned the presence of chromatophores (Bandaranayake & Gentien, 1982; Herring, 1972; Vestheim & Kaartvedt, 2006; asterisk-shaped red

patches on Figure 2I), it is not clear whether copepod species have actually developed cells or organs dedicated to pigment production, similar to what has been demonstrated in the liver for birds (Mundy et al., 2016). Intensity or localization of pigmentation can also help identifying species: the recognition of red rather than red-orange pigmented individuals from the genus *Neocalanus* in live collections led to the discovery of a new *Neocalanus* species from the subarctic Pacific Ocean (Miller, 1988), and the redness of antennas and genital somites can be used to distinguish two *Calanus* congeners (*C. finmarchicus* and *C. glacialis*; Choquet et al., 2018; Lindeque et al., 2022; Nielsen et al., 2014).

Two types of carotenoids are found in copepods: astaxanthin and canthaxanthin. Thirty-two studies provide a detailed description of carotenoid pigments' molecular forms for various species and developmental stages (170 measurements). In 96% of these measurements, astaxanthin was the dominant carotenoid (mentioned as "dominant" in the text or as indicated by a contribution of more than 50%). In only six cases, astaxanthin was in minority (<50%), while a mix of other phytoplanktonic

A : *Leptodiaptomus minutus*, **Canadian lake in winter (A₁) and summer (A₂),** Grosbois and Rautio (2018)
B : *Diaptomus castor*, **fishless pond in Sweden,** Brüsin et al. (2016)
C : *Eudiaptomus gracilis*, **lake in Sweden,** Hylander et al. (2012)
D : *Undinela vulgaris,* **equatorial Atlantic at day (D1), and night (D2),** Kiko et al. (2020) (personal observation)
E : *Tigriopus californicus*, **female with eggs (E1), mating adults (E2), US west coast,** Powers et al. (2019) and Tsuboko-Ishii and Burton (2018)
F : Unknown species, **blue hole in the Bahamas,** Lee et al. (2019)
G : *Calanus sp*, **Arctic seas**, Choquet et al. (2018)
H : *Pontella fera*, **equatorial Atlantic ocean,** Rahlff et al. (2018)
I : *Pareuchaeta norvegica*, **Norwegian coast,** Vestheim and Kaartvedt (2006)

FIGURE 2 Examples of pigmented copepods from freshwater (A–C) and marine (D–I) ecosystems from the literature. Images were all taken from published articles, indicated on the figure. Carotenoids can be accumulated throughout the body in yellow-red (B, C, D2, E2, I) or blue (D1, H) hues. Also, they can be localized in specific structures such as lipid droplets and sacs (A1, G), eggs (A2, E1), antennules (G, H), or genital somite (G).

carotenoid molecules (β-carotene, chlorophylls *a* and *b*, echinenone, and β-doradexanthin) from the diet contributed the most (Bandaranayake & Gentien, 1982; Lotocka, 2004; Moeller et al., 2005; Rautio et al., 2009; Rhodes, 2007). Canthaxanthin was found in only 21 samples of copepods, and always at low levels (traces, <5%). Astaxanthin is thus the dominant pigment in copepod species, as it is in noncopepod aquatic crustaceans (Matsuno, 2001). It has to be synthesized from dietary phytoplankton carotenoids (Babin et al., 2010; Caramujo et al., 2012; Moeller et al., 2005) and its bioconversion involves a series of ketolase and hydroxylase enzymes that oxidize β-carotene, lutein, or zeaxanthin into astaxanthin (Weaver, Cobine, & Hill, 2018). Parsimoniously, the most oxidized precursors from the diet will be favored. Three metabolic pathways coexist but most species, such as *Paraeuchaeta russelli* (Bandaranayake & Gentien, 1982), *Cyclops kolensis* (Czeczuga et al., 2000), *Acartia bifilosa*, and *Pseudocalanus acupes* (Lotocka, 2004), rely on echinenone and canthaxanthin as intermediates. It has recently been suggested that carotenoid bioconversion is linked to mitochondrial activity: the shared pathway

hypothesis (SPH) (Powers & Hill, 2021; Powers et al., 2022). It proposes that ketolations and hydroxylations of algal carotenoids (oxido-reduction reactions) are involving electron donors located in inner mitochondrial membranes (probably NADP and NADPH, see Powers and Hill, 2021 for detailed explanations). The SPH offers new perspectives on the link between astaxanthin production and copepod fitness, which will be further discussed in the third part of this review. However, while respective genes and associated membrane enzymes were identified in turtles and birds (Mundy et al., 2016; Twyman et al., 2016), only candidates for possible genes have been proposed for crustaceans like copepods (Mojib et al., 2014). Latest advancements on the molecular bases of astaxanthin synthesis are discussed in the fourth part.

In the animals, astaxanthin can either be found in a free form, esterified with fatty acids (mono- or diesters) or associated with a protein (Matsuno, 2001; Schneider et al., 2016). The proportion of each form can vary between species, but also at the individual level over time. Free astaxanthin can be incorporated into cell membranes where it preserves membrane structure by

reducing phospholipids' peroxidation (McNulty et al., 2007, see next paragraph). For the majority of copepod samples (61%) from a detailed dataset of astaxanthin forms (Data2), free astaxanthin is the dominant form. The remaining 39% preferentially accumulate astaxanthin esters. All samples with more than 70% of astaxanthin being esterified belong to copepod species known to accumulate large lipid storage (*Calanus finmarchicus*, *C. pacificus*, *C. helgolandicus*, *Arctodiaptomus walterianus*, and *Leptodiaptomus minutus*; Data2). In a one-year study, the amount of unesterified astaxanthin was rather constant in adults of *Leptodiaptomus minutus* (Schneider et al., 2016), but the proportion of specific esters can vary substantially in them. Starvation of *Calanus pacificus* population first led to an increase of free astaxanthin from esters degradation, followed by an overall decrease in total astaxanthin content (Juhl et al., 1996). These observations led authors to suggest a strong link between carotenoid pigmentation and lipid metabolism (Foss et al., 1987; Juhl et al., 1996; Lotocka, 2004; Schneider et al., 2016; Sommer et al., 2006). Lotocka (2004) formulated the hypothesis that astaxanthin forms vary with ontogeny, that is, that free forms are dominant in nauplii and young copepodites and esterified forms in adult stages. However, the limited number of quantifications of carotenoids according to the development stage does not allow for testing it statistically (Data2) (Cui et al., 2021; Hairston, 1979; Holeton et al., 2009; Lotocka, 2004). We suggest that ontogenic accumulation of astaxanthin esters may be especially important for species in which adults depend on lipid reserves for survival and reproduction, and for which the proportion of free astaxanthin and esters varies according to the individual state (reproductive stage, starvation, etc.) (Juhl et al., 1996; Schneider et al., 2016).

Occasionally, the formation of a caroteno-protein complex with astaxanthin or canthaxanthin induces a blue coloration (Cheesman et al., 1967; Mojib et al., 2014). This phenomenon was observed, but rarely quantified, in the freshwater copepod *Eudiaptomus amblyodon* (Czeczuga, 1975) and in many marine pontellid and calanoid copepods. A uniform blue coloration (Figure 2H) is particularly common in neustonic copepods from equatorial waters and was described for the following species: *Labidocera glauca* (Smith, 1941), *Labidocera nerrii*, *Labidocera woolastoni*, *Labidocera acutifrons*, *Pontellopsis regalis*, *Pontellopsis villosa*, *Anomalocera patersoni* (Herring, 1972), *Pontella fera* (Herring, 1965; Rahlff et al., 2018), *Pontella valida* (Venkataramana et al., 2017), *Pontella sinica* (Ory et al., 2017), *Undinula vulgaris*, *Centropages furcatus* (Bandaranayake & Gentien, 1982), *Ivellopsis denticauda* (Rahlff et al., 2018), *Acartia fossae* (Mojib et al., 2014), *Acartia eurythreaea* (Nakajima et al., 2013), *Farranula gibbula* (Medellín-Mora et al., 2021), *Saphirinna* sp.

(Ory et al., 2017), and *Corycaeus* sp. (Mojib et al., 2014; Ory et al., 2017). Recently, observations of *Undinula vulgaris* transitioning from a blue to a red coloration between day and night were made in Atlantic waters (Figure 2D1,D2 provided by R. Kiko; Kiko et al., 2020) and raises many questions about the metabolic control of carotenoid complexation with proteins. Furthermore, planktonic ecologists often observe bright red adults with blue eggs in *Paraeuchaeta* spp. and *Euchaeta* spp. (e.g., Bandaranayake & Gentien, 1982; Lee et al., 1974; Vestheim et al., 2005). This intriguing phenomenon shows that astaxanthin can be found in different forms between the copepod body and the eggs, which also implies a link between astaxanthin and ontogenic development.

Physiological action modes of astaxanthin

The strong antioxidant power of astaxanthin is a unique property that could explain its ubiquity in copepods. To understand the molecular functions underlying this property, we provide a succinct description of its reactions with ROS at the molecular level (Figure 3). ROS are oxidant molecules, mainly produced by endogenous metabolism such as mitochondrial respiration, or sometimes induced by external stressors such as ultraviolet radiation (Krumova & Cosa, 2016, details on Figure 3A). In animals, the development of a complex antioxidant system was selected through evolution to avoid critical ROS damage on proteins, lipids, and DNA (Figure 3B), which is the main cause of cell aging (Finkel & Holbrook, 2000). However, oxidative stress can still occur when enzymatic and nonenzymatic antioxidant defenses are not efficient enough to neutralize all ROS (Monaghan et al., 2009). The astaxanthin molecule is an important part of this defense system since its long-conjugated carbon chain has a particularly strong antioxidant power (Miki, 1991).

The antioxidant capacity of astaxanthin has two components represented in Figure 3C: a strong quenching effect against singlet oxygen and a scavenging effect against free radicals (Miki, 1991; Naguib, 2000). In singlet oxygen quenching, the energy of an excited oxygen molecule ($^1O_2$) is transferred to the carotenoid by direct contact, and the excessive energy is relaxed, thanks to the vibration of the carbon chain. The astaxanthin activity for neutralizing singlet oxygen is approximately 10 times stronger than those of other carotenoids (Miki, 1991). Astaxanthin is also very efficient to scavenge free radicals, that is, molecules with unpaired electrons (Naguib, 2000; Terao, 1989). In particular, it can trap lipid radicals (LOO•) produced in large quantities when hydroxyl radicals (OH•) degrade lipids through a chain
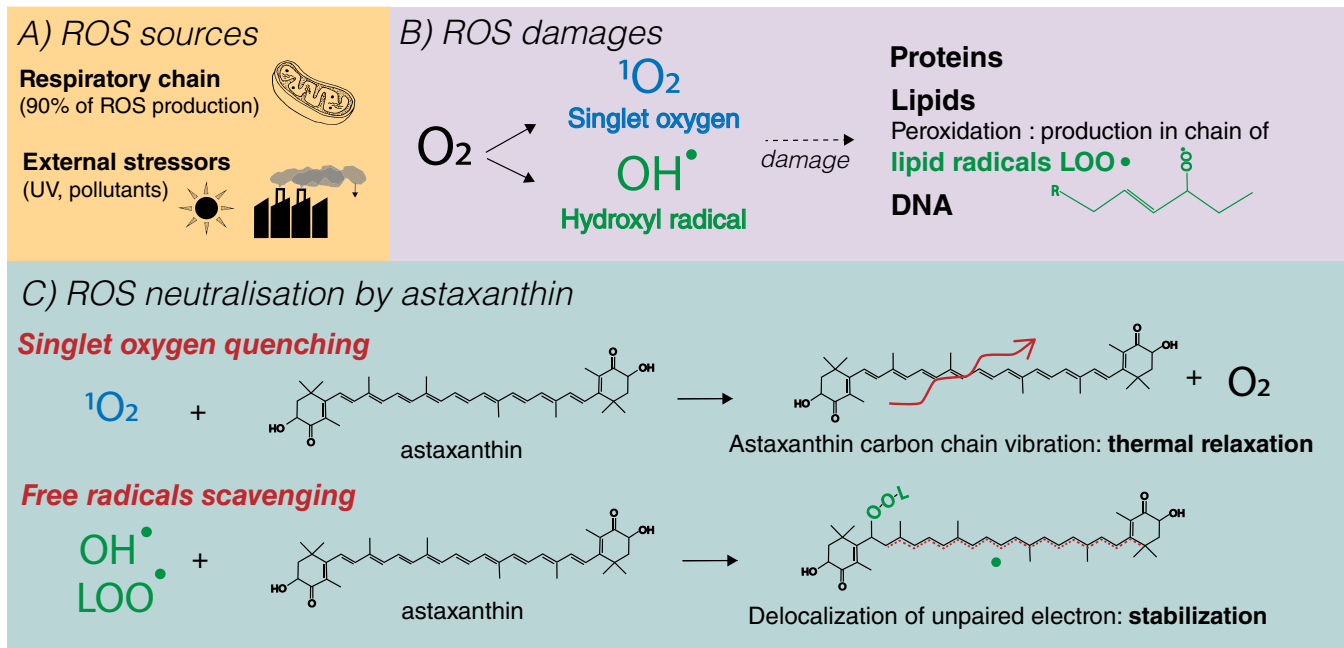
FIGURE 3 Reactive oxygen species (ROS): sources, damages, and neutralization by astaxanthin. (A) ROS are produced by active oxic metabolism and/or external stressors. (B) Free radicals (atoms or compounds with one or more unpaired electrons, noted with the bullet symbol (•, in green) and nonradical oxidants (atoms or compounds in an excited electronic state at high energy level, in blue) can induce damages on proteins, lipids, and DNA. Note that lipid peroxidation by hydroxyl radicals (OH•) can lead to the chain production of lipid radicals (LOO•), also dangerous for cell components. (C) Thanks to its long double-bonded carbon chain, astaxanthin can buffer singlet oxygen by quenching, or buffer free radicals by electron scavenging. This scheme is a synthesis of the information found in Balaban et al. (2005), Girotti (1985), Krumova, and Cosa (2016) for sections A and B, and in Miki (1991), Naguib (2000), and Terao (1989) for panel C.

reaction ("lipid peroxidation"; Girotti, 1985). The product of the reaction between the radical (OH• or LOO•) and astaxanthin is stable and stops the chain reaction of lipid degradation (Terao, 1989). While the existence of these two mechanisms could explain the prevalence of astaxanthin in copepods, the precise environmental conditions favorable for astaxanthin accumulation are still unclear. In the next part, we discuss the various biotic and abiotic forcings fostering copepod pigmentation.

EFFECTS OF ABIOTIC AND BIOTIC FORCINGS ON ASTAXANTHIN CONCENTRATIONS IN COPEPODS

Environmental controls on pigmentation

Among the most studied environmental variables that correlate with carotenoid pigmentation in copepods are lake elevation, latitude, temperature, and solar radiation (Figure 4; Byron, 1982; Hairston, 1979; Hansson, 2000; Siefken & Armitage, 1968). It has been recognized for a long time that copepods from high-altitude lakes are the reddest (Figure 4M). Indeed, we found the two highest concentrations of astaxanthin in copepods from lakes at

4890 m (17.2 µg mg DM^{-1}; Sommaruga, 2010) and 3613 m high (8.5 µg mg DM^{-1}; Persaud et al., 2007). Including all freshwater measurements, we found a significant correlation between astaxanthin concentrations and lake elevation ($n = 65$; $p < 0.01$; Appendix S1: Figure S2), but with a high variability ($R^2 = 0.12$). In marine ecosystems, three studies showed that latitude is positively correlated with carotenoid content at a regional scale: arctic and subarctic species are more pigmented than tropical and temperate species (Figure 4I) (Hansson, 2000; Hansson et al., 2007; Hylander et al., 2009). However, when using the complete dataset assembled here, there is no significant correlation between latitude and carotenoid content at a global scale (Appendix S1: Figure S3). This highlights how difficult it is to reveal a large-scale trend for a physiological trait that can be finely regulated at relatively short temporal and thus small spatial scales (see *The need for a mechanistic comprehension of a very plastic trait*). Moreover, studies were performed in different seasons and for various species, depths, and ecosystems. Therefore, it is likely that this dataset may not be exhaustive and representative enough to reach a statistical signal stronger than the effect of all these sources of variability.

Furthermore, we cannot regard the effects of altitude or latitude as independent factors of pigmentation, but
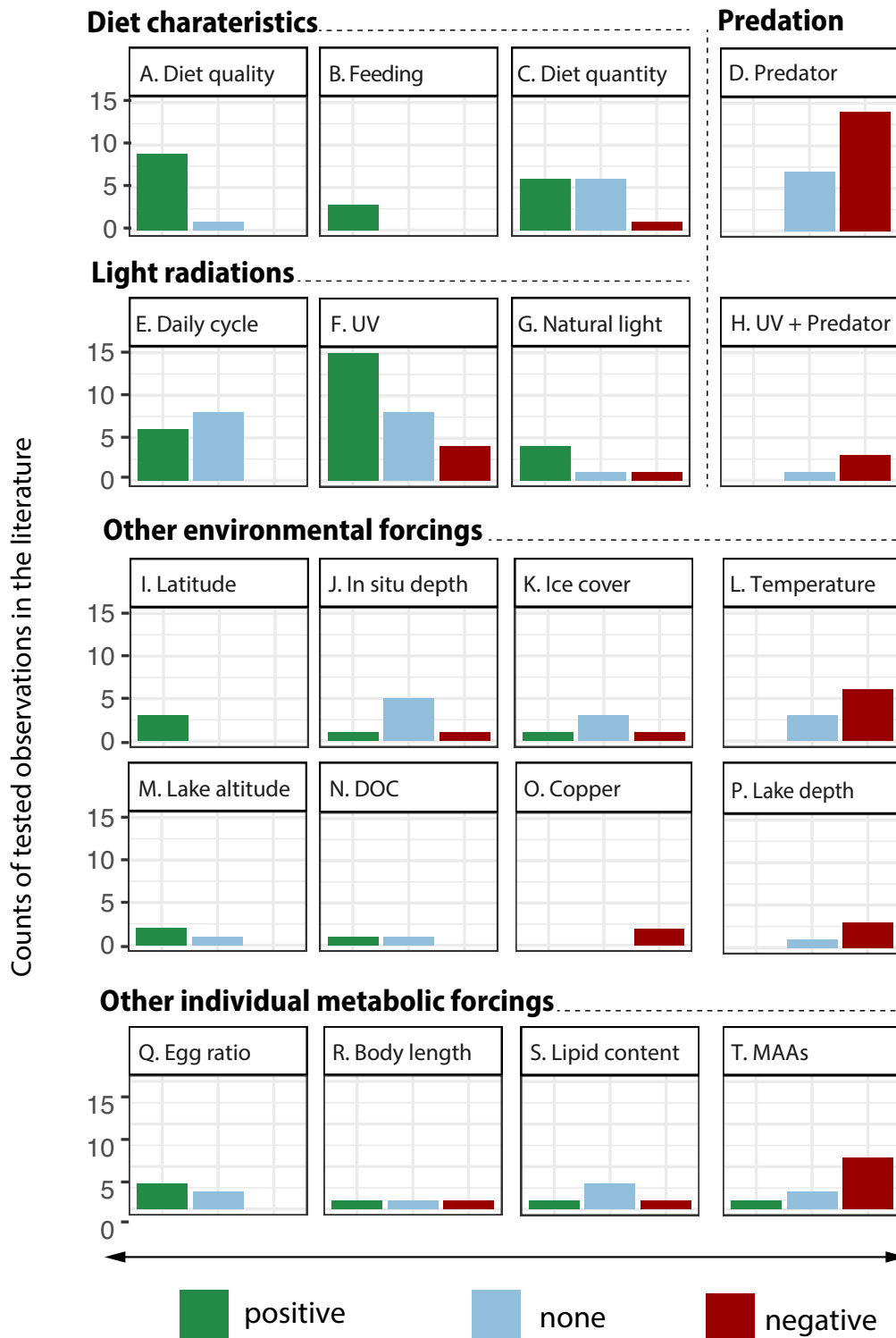
Correlation of various forcing variables with individual carotenoid content

FIGURE 4 Counts of correlations between copepod carotenoid pigmentation and main environmental, biological or ecological forcings tested in the literature. All correlations between the carotenoid content of a copepod sample and a forcing variable (grouped in the categories: diet, predation pressure, light radiation stress, and other environmental or metabolic forcings) were retrieved from the literature (Data3: https://doi.org/10.5281/zenodo.7671742). If statistically significant, the correlation was reported as "positive" (green) or "negative" (red). If not significant, the correlation was reported as "none" (blue). Only forcing variables with at least two tested correlations reported were considered (in situ or experimental). MAAs, mycosporine-like amino acids; UV, ultraviolet radiation (see Box 1).

we rather emphasize their relation with direct drivers such as temperature, solar radiations, and ice cover. Solar radiation, especially short wavelengths (ultraviolet), enhances astaxanthin accumulation in most cases (Figure 4F,G). It can explain why copepods living in high-altitude ecosystems are especially red, in particular if lakes or ponds are shallow and do not provide refuge at greater depths (see negative correlation between carotenoid content and lake depth, Figure 4P). Similarly, the highest carotenoid concentrations in marine copepods, on par with their freshwater counterparts (Appendix S1: Figure S1), were observed for *Tigriopus* sp. living in shallow supralittoral tidal pools subjected to strong solar radiation (Davenport et al., 2004; Weaver, Wang, et al., 2018). This confirms the expected antioxidant role of astaxanthin against UV-induced ROS. However, two observations contradict this hypothesis: (1) most marine copepods living under high solar radiation at low latitudes do not accumulate red pigments at all, or only very little (Figure 2F) (Lee et al., 2019); (2) some populations found in arctic lakes covered by ice in winter are bright red, even though light penetration is severely limited (Figure 2A) (Grosbois & Rautio, 2018). Therefore, the protection against light and UV radiation cannot be the only explanation for redness variability, especially because UV is strongly attenuated in aquatic ecosystems (Tedetti & Sempéré, 2006).

Temperature is a key variable as it is almost always negatively correlated with carotenoid content in vivo (Figure 4L), and arctic freshwater and marine copepods are known to show intense red colors (Byron, 1982; Garcia et al., 2008; Schneider et al., 2016; Siefken & Armitage, 1968; Snoeijs & Häubner, 2014). This observation, in addition to in vitro experiments on astaxanthin molecules (Pedersen et al., 2014), suggests that astaxanthin is likely to be more stable at cold temperatures than at warm temperatures. As a consequence, the lack of red pigmentation in tropical environments with strong solar radiation may be caused not by a lack of usefulness of astaxanthin molecules, but rather by molecular instability. Nevertheless, if redness is much scarcer in intertropical waters, blue color induced by astaxanthin complexation with protein is common for copepods living in the first centimeters under the surface (Rahlff et al., 2018). This stability of the various forms of astaxanthin (including caroteno-proteins) according to temperature would be interesting to investigate further, along latitudinal gradients in marine ecosystems (Table 2).

As a conclusion, solar radiation and temperature are both key variables explaining pigmentation variations at a global scale, but are not sufficient to solve the color puzzle alone. In the next paragraphs, we discuss the effects of internal metabolism (diet and lipid synthesis) and external interactions (predation and camouflage) that also play an important role in copepod individuals' pigmentation.

Biological and metabolic drivers of pigmentation

Copepods rely on phytoplankton precursors to synthesize astaxanthin (Weaver, Cobine, & Hill, 2018). As a consequence, variations in their phytoplanktonic diet and feeding activity have a significant impact on astaxanthin production (Figure 4A–C). A minimum of algal carotenoids is a sine qua non condition for copepods to build astaxanthin stocks: the harpacticoid *Tigriopus* spp. loses its red color with a yeast diet lacking carotenoids (Davenport et al., 2004; Powers et al., 2019; Weaver, Cobine, & Hill, 2018). An example of calanoid copepods from the Baltic Sea shows that astaxanthin production in mesocosms was highest when the population was fed on a diverse phytoplankton community dominated by chlorophytes, dinoflagellates, and diatoms with thin silica frustules in comparison with a diet composed of a low diversity of heavily silicified diatoms (Andersson et al., 2003). Diet quality (composition and diversity) is thus an undeniable driver of astaxanthin production (Figure 4A; Andersson et al., 2003; Rhodes, 2007; Van Nieuwerburgh et al., 2005).

Because algal communities strongly vary in terms of abundance and composition over time, it can be expected to see equivalent variations of astaxanthin accumulation in copepods. Indeed, for 33 of 37 time series, astaxanthin content showed seasonal patterns with maxima in winter (45%) or in spring (40%) (Data1 and code, "seasonal pic" column). However, the correlation with food quantity is not always obvious (Figure 4C) and pigmentation can vary a lot among copepods living under the same food conditions (Hansson, 2004). Holeton et al. (2009) showed that the astaxanthin production first increases linearly with increasing food concentration, but drops for algal concentrations (*Tetraselmis suecica*) higher than 150 µg C L^{-1} because of inefficient assimilation at too large food quantity, which implies that there is an optimal food concentration for astaxanthin production.

Redness and phytoplanktonic dynamics can also be fully decoupled, resulting in astaxanthin maxima in the cold, less productive season (Davenport et al., 2004; Hairston, 1979). In the total darkness or in some turbid waters, some copepods are still bright red (Schneider et al., 2012, 2016; Trudnowska et al., 2022). As discussed above, pigments have long been known to be associated with lipids in "fat cells" (Ringelberg & Hallegraeff, 1976; Siefken & Armitage, 1968) or in lipid droplets (Grosbois & Rautio, 2018; Veen, 2005). In this context, the most likely function of astaxanthin is to scavenge lipid peroxyl radicals (Table 1, H4), inhibiting chain reactions of lipid degradation because of oxidative stress (Figure 3C). Astaxanthin, which has a similar molecular length as fatty acids, can be incorporated into cell membrane bilayers where it

preserves structure and intercepts radicals through the entire membrane (McNulty et al., 2007; Woodall et al., 1997). When esterified with fatty acids, astaxanthin could help diapausing and/or capital-breeding species to protect their long-term energy deposits to survive rough and variable environments (Foss et al., 1987; Juhl et al., 1996; Schneider et al., 2016; Sommer et al., 2006). More studies are, however, still needed to clarify the link between lipids and astaxanthin accumulation, because reported results are not consistent (Figure 4S). The unique and very detailed study on the boreal copepod *Leptodiaptomus minutus* from Lake Simoncouche, Canada, showed a strong seasonal correlation between astaxanthin and lipid content, with maximum accumulation of astaxanthin in late fall at the peak of lipid anabolism (Schneider et al., 2016).

Consistently among all the experiments, the periods of net loss in astaxanthin coincided with peaks of egg production in spring and summer, leading to minimum astaxanthin contents. There was a clear transfer of pigments from lipid stores to eggs, leading to new questions and hypotheses (Figure 2A) (Grosbois & Rautio, 2018). Observations of red pigments in eggs and spermatophores have first been made in 1979 (Hairston, 1979; Holeton et al., 2009; Schneider et al., 2017). A general positive correlation appears between carotenoid content and egg ratio (Figure 4Q). In particular, eggs of *Tigriopus sp.* transit from dark gray to red as embryos develop (Weaver, Cobine, & Hill, 2018), and astaxanthin of *Euchaeta japonica* blue eggs is apparently metabolized by early nauplii stages, who progressively become colorless with development (Lee et al., 1974). Astaxanthin accumulation in eggs and nauplii suggests an important role in early copepod development (Table 1, H_5). For example, it could protect buoyant eggs that stay in surface layers and are then exposed to higher UV radiation without the possibility of vertical migrations to avoid them (Browman et al., 2000; Hairston, 1979). As suggested by Lotocka (2004), a broad-based role of astaxanthin in juveniles is conceivably to fight against ROS produced by yolk catabolism and rapid growth (Table 1, H5), since most ROS are produced by normal metabolism and not by external stressors (Figure 3A). However, further studies are needed to link astaxanthin content to hatching success and nauplii survival (Table 2).

Impacts of intra- and interspecies interactions on pigmentation

The role of astaxanthin pigments cannot be reduced to antioxidative protection, especially considering that the main function of color in nature is to trigger behavioral responses and interactions (as for birds, flowers, and fish). The accumulation of red pigments makes copepods more conspicuous in well-lit waters, increasing the risk of capture by visual predators (Hairston, 1981). Some studies showed that copepods accumulate more pigments when they inhabit fishless freshwater environments (Byron, 1982; Hansson, 2000; Luecke & O'Brien, 1981). The comparison of mean carotenoid concentrations between lakes with and without predation pressure by fish (with predators: 2.06 μg mg DM^{-1}, $n = 34$; without predators: 3.35 μg mg DM^{-1}, $n = 22$) was significant using the data collected for this review (Wilcoxon rank sum test, $p = 0.03597$; Appendix S1: Figure S4). This result suggests again the influence of predation pressure on carotenoid pigmentation. It has, however, to be noted that such habitats are often shallow ponds that freeze up or evaporate seasonally and where copepods are subjected to strong radiation, which also promotes coloration (Appendix S1: Figure S4, points colored according to altitude). Other studies showed that copepods are able to adjust the level of pigmentation according to fish threat in lakes (Figure 4D) (Hansson, 2004; Lee et al., 2019; Schneider et al., 2016). In vitro, pigmentation is reduced a few days or weeks after perceiving chemical cues of predators (Brüsin et al., 2016; Hansson, 2004; Hylander et al., 2012; Veen, 2005). These observations indicate that astaxanthin synthesis and degradation are fine regulated at the cellular level according to predation pressure (see *The need for a mechanistic comprehension of a very plastic trait*).

Another zooplankton strategy to avoid visual predation is diel vertical migration (DVM). By spending time at depth, copepods diminish their probability to be targeted by visual predators in brighter surface waters. Furthermore, because light wavelengths from 600 to 750 nm are absorbed in the upper few meters by water molecules, red color is the optimal coloration for zooplankton crypticity at depth (Johnsen, 2002, 2005). When deep visual predators use green-blue "bioluminescent searchlights," red animals appear black and are completely hidden. Also, we can observe a color gradient from blue and transparent animals in surface layers to red in the deep waters (Johnsen, 2014). Accumulating red pigments, and thus being able to avoid predators at greater depths, could represent a selective advantage for vertically migrating copepods (i.e., *Paraeuchaeta noervegica*; Vestheim et al., 2005) or for copepods spending a large part of their life at great depths (i.e., diapausing *Calanus* spp.; Daase et al., 2021; Kvile et al., 2019). According to Hays et al. (1994), large copepods are performing more extensive DVM than smaller taxa, but pigmentation is correlated with DVM only for the small (<1 mm) copepod taxa. As size and color are the main factors influencing visibility, authors argued that only small species can afford to accumulate pigmentation without increasing their chance

to be captured when residing in surface layers. However, the largest copepod species in the oceans (*Calanus hyperboreus*) is strongly pigmented and migrates vertically. In general, most published data of copepod redness associated to position in the water column do not provide evidence for depth-dependent pigmentation (Figure 4J).

The intensity of the color or the color itself might also change according to visibility conditions. Vestheim and Kaartvedt (2006) showed that copepods were redder by night than by day, presumably avoiding visual predation in brighter waters. Blue color, on the other hand, is mostly constant for neustonic copepods (Figure 2H) and represents an antioxidant defense against ultraviolet radiation (Herring, 1972; Nakajima et al., 2013; Venkataramana et al., 2017), in addition to a camouflage from fish and birds in surface waters. Indeed, their blue reflectance perfectly matches water color (Rahlff et al., 2018), which might be advantageous for some blue copepods performing reverse vertical migrations (Chae & Nishida, 1995; Tester et al., 2004). Recent observations of copepods transitioning from blue during the day to red at night (Figure 2D1,D2; R. Kiko, personal observation, 2012, 2013) offer new research questions: could color change according to day cycle and/or depth to provide a defense against visual predation? With the exception of blue neustonic population or red population living in shallow tidal pools or ponds (Cui et al., 2021; Davenport et al., 2004; Sommaruga, 2010) (Figure 5), it could be argued that the use of astaxanthin as UV-protectant may be limited in deep lakes and offshore ocean because UV are absorbed in the few first meters (Tedetti & Sempéré, 2006) and large refuge depths are available. In those contexts, color might be evolutionary selected for camouflage against predators and/or for its general antioxidant properties, rather than UV protection (Lotocka, 2004; Schneider et al., 2016). One counterexample is the surface swarms of *Calanus* copepods showing a red color presumably used as a UV-protectant (Basedow et al., 2019). This is supported by the fact that the same species are transparent when they inhabit really turbid glacial waters (Trudnowska et al., 2022). However, the only trial of correlation between astaxanthin content and light radiation was not successful (Hylander et al., 2015). Carotenoids could help copepods against UV when they reside in surface layers, but only as one of the several functions that the astaxanthin "Swiss army knife" allows (Figure 5).

As a general conclusion, astaxanthin synthesis is performed preferentially in cold environments, with an optimal diet quality and quantity and limited predation pressure. Then, its accumulation depends on light stress, mycosporine-like amino acids (MAAs) synthesis possibility (Box 1), lipid metabolism, and egg production in females. The potential advantages conferred by pigmentation in terms of fitness are discussed hereafter.

HOW DOES REDNESS IMPACT COPEPOD FITNESS (REPRODUCTION, GROWTH, AND SURVIVAL)?

After exploring the mechanisms triggering carotenoids production and the metabolic pathways involved, we estimated the effects of carotenoid content on individual fitness metrics such as percentage of survival, number of eggs produced or metabolic activity indices (Data4). We focused this analysis on redness because fitness variables were only tested on red copepods in the literature, even if blue pigmentation appears to be a clear advantage for neustonic animals (Rahlff et al., 2018). We found that red pigmentation is mostly beneficial for the copepods (Figure 6). Of 50 statistically tested effects on one fitness variable, red pigmentation was favorable in 31 cases (62%), neutral in 11 cases (22%), and unfavorable in 8 cases (16%). The unfavorable effects are all linked to visual predation selectivity (Figure 6). When red copepods were incubated with predators, which was always done in experimental settings and under white light (i.e., no tests with attenuated light typical of deep waters), they were preferentially preyed upon, and their probability to survive was lower than that of their transparent counterparts (Byron, 1982; Gorokhova et al., 2013; Hairston, 1976, 1979; Luecke & O'Brien, 1981). However, redness was beneficial when measuring other survival metrics. The most repeated experiment was the survival (or mortality) test for transparent and red morphs that were exposed to visible and UV radiation (Byron, 1982; Caramujo et al., 2012; Davenport et al., 2004; Hairston, 1976; Luecke & O'Brien, 1981; Ringelberg & Hallegraeff, 1976; Tartarotti, 1999), or that were exposed to copper or other pro-oxidant agents (Caramujo et al., 2012; Weaver, Cobine, & Hill, 2018). In 71% of these experiments, redness was an advantage, presumably due to astaxanthin antioxidant activity. Molecular indicators such as hsp70 gene regulation and aconitase activity showed that carotenoid-rich morphs presented less oxidative stress levels than transparent ones (Tartarotti et al., 2018; Weaver, Cobine, & Hill, 2018). Coherently, red copepods expressed a better antioxidant capacity, inferred by Glutathione S-transferase (GST) activity, oxygen radical antioxidant capacity, and trolox equivalents (see Figure 6; Gorokhova et al., 2013; Hylander et al., 2012; Tartarotti et al., 2018). Complementary results on survival also showed positive effects (Figure 6), for example, red copepods suffered less from parasite infestations (Veen, 2005).
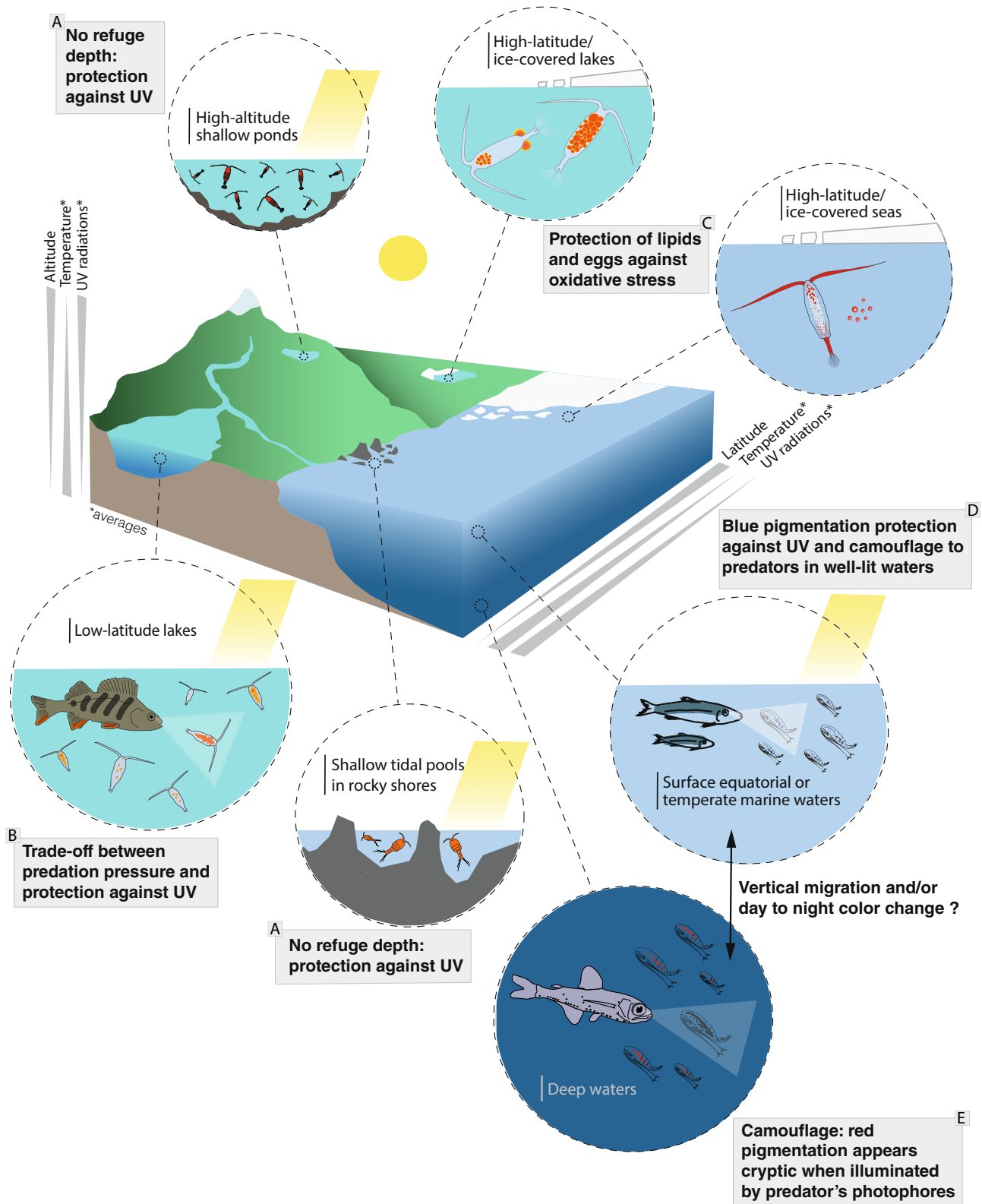
FIGURE 5 Carotenoid-based pigmentation as a Swiss army knife for copepod fitness in aquatic ecosystems. This figure summarizes the multiple roles that astaxanthin pigmentation can have for copepods in aquatic ecosystems at a global scale. For various latitudes, altitudes, temperature, and ultraviolet radiation (UV) conditions, red or blue pigments can improve copepods' fitness. Due to its antioxidant properties, it can buffer oxidative stress induced by UV (A, B, D), or by internal metabolic byproducts or pollutants such as metallic ions. In particular, astaxanthin can protect lipids reserves and eggs (C), which benefits diapausing and capital-breeding species. Finally, color variations can impact ecological interactions, increasing or decreasing prey visibility to visual predators in well-lit (B, D) or dark waters (E). Carotenoid-based pigmentation is a widespread and plastic functional trait in copepods, providing indirect benefits for aquatic trophic networks by the transfer of antioxidants, up to humans.

BOX 1   Carotenoids and mycosporine-like amino acids (MAAs).

A striking example linking environmental, physiological, and ecological drivers influencing antioxidant defenses.

MAAs are other photoprotective substances absorbing UV radiations. Their synthesis also depends on phytoplanktonic food availability (biological control), but with precursors different from carotenoid ones (Moeller et al., 2005). MAA accumulation is also triggered by light stress (environmental control); however, those molecules absorb at wavelengths between 310 and 360 nm (UV) and are not visible to predators in natural light. Consequently, MAAs do not enhance the risk of visual predation and are likely beneficial for individual fitness (ecological control). Studies that quantified both MAA and carotenoid contents often found a strong negative correlation between the abundances of these two compounds (Figure 4T), meaning that one type of photoprotective molecule will be favored by individual metabolisms (Hylander et al., 2009; Moeller et al., 2005; Persaud et al., 2007). Hylander et al. (2014) suggested that the generally smaller amount of carotenoid contents in marine ecosystems is due to a greater visual predation pressure, and a resulting preferential accumulation of MAAs. Yet, the difference of predation between freshwater and marine ecosystems has still to be proven. Nonetheless, it is likely that in very transparent tropical waters, MAAs presence would be especially valuable for fitness. In arctic ecosystems, MAAs synthesis was shown to be triggered by spring ice melting and consequent light stress for *Calanus* spp., whereas carotenoids are present, in lower but stable concentrations (Hylander et al., 2015). Could carotenoids and MAAs have complementary roles in these copepod polar species?
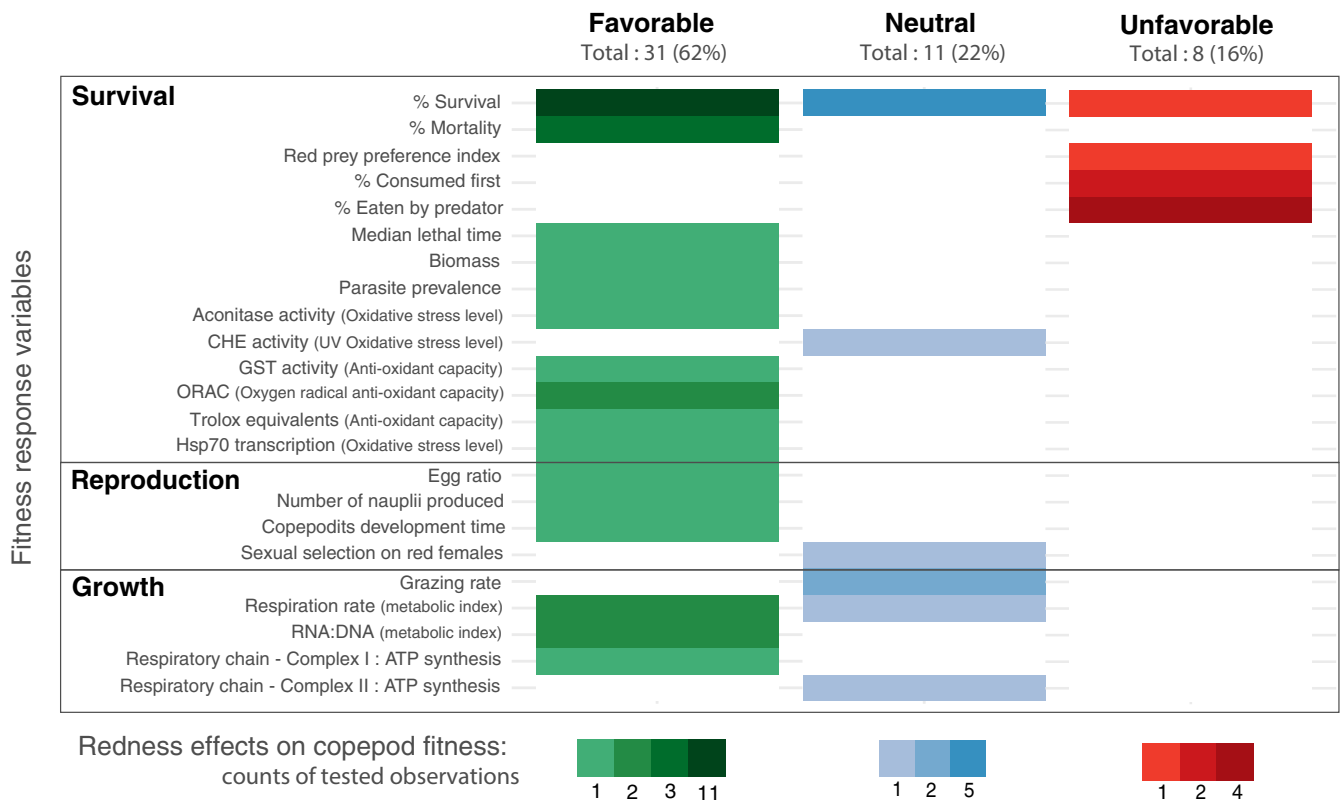
FIGURE 6 Carotenoid pigmentation effects on reproduction, growth, and survival of copepods. From the literature, we compiled every correlation that was performed to link carotenoid pigmentation and a fitness response variable. Thus, each count on the figure quantifies how accumulation of red pigments impacts fitness in colored copepods, compared with copepod lacking carotenoids: positive correlation, favorable (green); negative correlation, unfavorable (red); no correlation, neutral (blue). Fifty correlations were used to create this figure, according to statistics strength or to method's quality as discussed by papers' authors (see "comments" column in Data4 at https://doi.org/10.5281/zenodo.7671742 for more details). CHE, cholinesterase; GST, Glutathione S-transferase; Hsp, heat shock protein.

As the redness trait provides advantages (e.g., antioxidant protection) at some costs (e.g., increased visibility to predators), trade-offs must exist at the individual level. When copepods were exposed to predator cues, their pigmentation decreased independently from ambient UV levels, suggesting that predation is treated as a more severe or immediate threat than energy-rich radiation (Hylander et al., 2009). To compensate for lacking UV protection, however, these copepods counteracted UV-induced oxidative stress by doubling the activity of the GST enzyme. The level of pigmentation can thus be considered as a plastic defense trait, which can be induced when needed (Hansson, 2004; Hylander et al., 2012).

Color is also found to play a role in reproduction, which is a major measure of fitness (quantified by the number of descendants). The amount of eggs produced by females of the reddish marine *Eurytemora affinis* was significantly higher than their nonpigmented counterparts for the same food intake (Gorokhova et al., 2013) (Figure 6). When the freshwater species *Diaptomus* and *Leptodiaptomus* cannot produce carotenoids (neither MAAs, see Box 1), they produce less offsprings, which supports the hypothesis that, in absence of photoprotective agents, oxidative stress reduces reproductive success (Hylander et al., 2009) (Figure 6). In one experiment, astaxanthin concentration was also negatively correlated with ontogenic development time: strong pigmentation allowed for a faster development of copepodites (Powers & Hill, 2021). Molecular experiments are still required to fully elucidate the role of pigmentation in reproductive success (Table 2).

Even if sexual selection is mostly done through a developed chemosensory system or via mechanical cues in copepods (Ohtsuka & Huys, 2001), another hypothesis is the role of coloration as a signal of individual quality when choosing a mate ("honest signal"; Powers & Hill, 2021; Powers et al., 2019). This would imply that copepods are able to assess the color of their potential partners. Most recent studies showed that two types of eyes are present in copepods (Porter et al., 2017): a simple naupliar eye shared by the majority of species, and a more complex eye apparatus with a lens specific to Pontellid and Saphiirinid copepods (Land, 1988; Manor et al., 2009; Takahashi et al., 2015). The structure and functioning of copepods' eyes remain poorly understood, but a recent genomic approach found opsins genes in diverse taxonomical groups, probably associated to photoreceptors functioning like rods to distinguish light variations (Porter et al., 2017). This is coherent considering copepods' ecological behavior of vertical migration according to light (Cohen & Forward, 2002). In experimental studies, copepods are especially sensitive to blue and green light (Båtnes et al., 2015; Martin et al., 2005), but some surface Pontellids species have a broader wavelength sensitivity, including one pic at 600 nm (Cohen & Forward, 2002). Furthermore, polarization of light can be detected by copepods such as *Pontella karachiensis* or *Calanus finmarchicus* and is likely to be used to choose swimming direction or to perceive other individuals (Lerner & Browman, 2016; Manor et al., 2009). According to these various studies, it is almost certain that copepods are able to detect variations in light intensity and perhaps light polarization (Takahashi et al., 2015). However, as most copepods own photoreceptors sensitive to only one type of short wavelength, it seems unlikely that they are able to distinguish colors of their congeners for sexual selection, especially for astaxanthin-red color. Consistently, experiments have failed to demonstrate selection of mates according to red pigmentation (Figure 6; Powers et al., 2019). Nevertheless, for astaxanthin-blue copepods owning complex eyes with broader wavelengths sensitivity and living in surface bright environment, further experiments are needed to understand if mate selection mates can be done through light intensity, polarization and/or color variations.

Finally, it appears that individual growth (or metabolic capacity) is influenced by pigmentation (Figure 6). The first studies documenting the link between carotenoids and copepod fitness showed that respiration rate was higher in pigmented populations of *Diaptomus* spp. than in unpigmented ones (Byron, 1981). One explanation could be that the absorption of light by pigments leads to an increase of body temperature, which in turn stimulates the metabolism (metabolic stimulation hypothesis, Table 1, H2; Byron, 1981). This hypothesis was, however, criticized theoretically because of the small size of a copepod and the high thermal conductivity of water (Hairston, 1981), but not tested experimentally. Follow-up studies assayed metabolic activity from the RNA:DNA ratio and concluded that the higher metabolism observed in pigmented copepods (at constant grazing rate) was likely to reflect the higher antioxidant defenses that enabled individuals to increase metabolic rates without the associated oxidative costs (Garcia et al., 2008; Gorokhova et al., 2013). Recently, the SPH offers new perspectives on the link between astaxanthin concentrations and metabolic activity (Powers & Hill, 2021; Powers et al., 2022). As discussed in *Localization, molecular forms and metabolism of carotenoid pigments*, the SPH postulates that carotenoids bioconversion is linked to mitochondrial activity in the redox environment of the inner mitochondrial membrane. Powers and his colleagues (Powers & Hill, 2021; Powers et al., 2022) started to investigate the positive correlation between carotenoid concentrations and the functioning of the electron transport system in the mitochondria (ATP synthesis from Complex I and II; Figure 6). If mitochondrial activity does enhance carotenoids ketolation,

coloration could be considered as a true (honest) signal of individual fitness not because of an "ornamental resource allocation" linked to reproduction (as proposed for birds), but because color would be an honest indicator of the individual's "energetic capacity" (Powers & Hill, 2021). This hypothesis seems relevant for copepods, which probably do not use color for sexual selection (Powers et al., 2019). The SPH could, thus, explain why the coloration decreased strongly when copepods were exposed to copper in vitro (Figure 4O; Weaver et al., 2016) since copper disturbs the redox environment of the mitochondria. Impact on carotenoid's metabolism because of mitochondrial disturbance was again demonstrated recently (Powers et al., 2022). The decrease in coloration could also simply reflect pigments' degradation when used as antioxidants (Caramujo et al., 2012, Davenport et al., 2004). These various mechanisms are not mutually exclusive and all lead to the same general conclusion: redness is a good fitness indicator because it reflects a general antioxidant capacity (survival) and/or a good metabolic capacity (growth), beneficial for adults, eggs, and offspring (reproduction).

PERSPECTIVES AND IMPLICATIONS TO MONITOR AQUATIC ECOSYSTEMS IN A CONTEXT OF ANTHROPOGENIC PRESSURES

The need for a mechanistic comprehension of a very plastic trait

We have demonstrated that copepod pigmentation fluctuates at large seasonal and regional scales and that these variations are driven by abiotic and biotic factors (summarized in Figure 5). But copepod' color is also a very plastic trait that varies at small temporal and spatial scales (Trudnowska et al., 2020; Vestheim & Kaartvedt, 2006; Vestheim et al., 2005). It changes rapidly according to light cycles with a maximum pigment accumulation occurring at night when feeding activity is high, and decreasing with sunrise (Kleppel et al., 1985) or just after (Andersson et al., 2003; Ringelberg & Hallegraeff, 1976). In the laboratory, color can disappear in few hours after net sampling (E. Trudnowska, personal observation, 2018), and reappear when exposed to light radiation after short time periods (i.e., minutes, hours or days) (Garcia et al., 2008; Hansson, 2000; Moeller et al., 2005). In addition, a loss of color can be triggered within hours or days due to exposure to fish kairomones (Hansson, 2004; Hylander et al., 2012). To understand such rapid variations, correlative approaches are informative (Figure 4)

but not sufficient. According to Gorokhova et al. (2013), "*pigmentation could be an example of a genetic system that leads to multiple fitness peaks under multiple selection pressures*": a mechanistic understanding of the carotenoid metabolism using transcriptomics and proteomics approaches is still needed to dissect this genetic system (Table 2).

Current industrial applications of astaxanthin in human dietary supplement (Guerin et al., 2003; Ye et al., 2018) pushed genetic engineering to decipher and optimize its biosynthesis (Zhang et al., 2020). The enzymes involved in the astaxanthin biosynthesis pathway are well characterized in prokaryotes, protists, fungi, and plants. In animals, the investigation on the genetic basis of the carotenoid's bioconversion provided new arguments supporting the SPH (Powers & Hill, 2021). The first animal enzyme producing keto-carotenoids was identified by combining genetics and transcriptomics as a cytochrome p450 mono-oxygenase belonging to the family 2J19 (CYP2J19) found in the bird *Quelea quelea* (Lopes et al., 2016). Then, a CYP384A1 was identified in the red spider mite (Chelicerata) using a genetic approach (Wybouw et al., 2019). These two studies showed an elegant example of convergence evolution of cytochrome p450 to produce astaxanthin. More recently, homologs of CYP2J19 or CYP384A1 genes have been searched in orange-red decapods such as *Halocaridina rubra* (Weaver et al., 2020), *Neocaridina denticulata sinensis* (Huang et al., 2022), and *Exopalaemon carinicauda* (Jin et al., 2021), as well as in the copepod *Acartia fossae* (Mojib et al., 2014). They did not succeed in pointing to such homologs, but suggested other putative cytochrome p450 candidates. In the benthic copepod *Tigriopus californicus*, a sex-specific variation of astaxanthin content was found in hybrid versus parental lines and highlighted the link with the mitochondria and the presence of astaxanthin biosynthesis enzymes in copepods (Powers & Hill, 2021). Future works based on molecular cloning of ABP enzyme-coding genes or mutant analysis will definitely help to characterize the genetic and molecular basis of astaxanthin bioconversion in copepods and other crustaceans, because until now, DNA analyses have not shown any differences in genomes of pigmented copepods in comparison to unpigmented ones (Gorokhova et al., 2013). This absence of differences suggests that astaxanthin biosynthesis enzymes are maybe not present in copepods, but would come from the food (Lemoine & Schoefs, 2010). In microalgae, it was indeed demonstrated that astaxanthin production is enhanced by grazing-induced stress (Albini et al., 2019): in response to predation, astaxanthin biosynthesis enzymes could be expressed in phytoplankton and used for astaxanthin production in copepods. Notifying that the gut is often the

reddest part of the copepod, a last hypothesis could be that gut microbiota bacteria are performing the astaxanthin synthesis. Astaxanthin biosynthesis pathway enzymes have already been identified in several marine bacteria such as *Brevundimonas* sp. (Asker, 2017; Chae et al., 2021), and this bacterium is also a major component of the gut microbial community in certain copepods (Chae et al., 2021). Thus, molecular studies of copepod gut microbiota could help to identify the role of bacteria in astaxanthin accumulation. The SPH, the food, or the gut origin hypotheses of astaxanthin biosynthesis pathways are not mutually exclusive and fine-tuned processes may occur to control the astaxanthin bioconversion. To guide future research, Table 2 summarizes potential perspectives about copepods' astaxanthin pigmentation research, including the need of interdisciplinary studies between molecular biologists and aquatic ecologists.

Astaxanthin content in the context of anthropogenic pressures

Living in the Anthropocene (Zalasiewicz et al., 2021), aquatic organisms are subjected to multiple threats caused by human activities, often pushing them beyond their physiological tolerance limits and their ecological niches. We have shown that esterified forms of astaxanthin are preferentially accumulated with lipids in cold, and sometimes ice-covered waters; and we discussed the hypothesis that astaxanthin and its esters may not be as stable in warm as in cold environments. If this hypothesis is verified, an increase in global temperature with climate change could reduce astaxanthin content and consequently the individual fitness of copepods, particularly in polar keystone species such as the *Calanus* or *Leptodiaptomus* congeners (Schneider et al., 2016; Trudnowska et al., 2020). Accumulation and protection of lipid stores for an extended time, that is, during winter, are crucial for polar ecosystem food webs (Browman et al., 2000; Falk-Petersen et al., 2009; Record et al., 2018; Turner, 2015). Lower astaxanthin content in copepods could then have a negative impact on the entire aquatic food web of these polar environments. The link between carotenoid accumulation, lipid protection, and temperature needs to be better understood in a context of climate change (Table 2).

Lipids degradation by oxidative stress can also be caused by human industrial pollution and the amount of oxidant pollutants, including metallic ions, released by anthropic activities is astonishing (Amoatey & Baawain, 2019). As a first example, oil pollution induces lipid peroxidation in *Calanus finmarchicus* and a higher activity of their antioxidant enzymes is needed to compensate for such oxidative stress (Soloperto et al., 2022).

Astaxanthin accumulation (known to occur in this species) would be a useful additional help against such human pollutants. Copper, as a second example, is released by mining, industrial and domestic emissions, and is associated with chemical fertilizers and pesticides (Flemming & Trevors, 1989). This pollution could contribute to the degradation of astaxanthin molecules or to the inhibition of astaxanthin production by modification of the mitochondrial redox state (Caramujo et al., 2012). On the other hand, some *Tigriopus* species, often bright red, have shown to be very tolerant to copper pollution in contrast to other organisms in coastal areas (Medina et al., 2008), showing the increased resilience enabled by astaxanthin pigments.

Last but not least, astaxanthin is accumulated when the phytoplanktonic diet is diverse (Andersson et al., 2003; Rhodes, 2007; Van Nieuwerburgh et al., 2005) and available in optimal—meaning not too abundant—quantities (Holeton et al., 2009). Excessive nutrient inputs leading to eutrophication of aquatic environments, with typical intense single-species phytoplanktonic blooms, could then be detrimental for astaxanthin copepod production.

Redness as a fitness index for copepod communities and aquatic food webs

Even if the ultimate mechanisms responsible for carotenoids' accumulation in copepods are not yet completely understood, our current state of knowledge allows us to consider astaxanthin pigmentation as a "Swiss army knife" for individual fitness in copepods (Table 1, H8; Figure 5). As a concrete example, *Calanus finmarchicus* and *C. glacialis* seem more pigmented when they inhabit their preferred water masses (i.e., Atlantic and Arctic, respectively), showing that color could be a fitness indicator when individuals are apparently in the best conditions to bioconvert carotenoids ("in their comfort zone": Trudnowska et al., 2020). The trait-based approach to aquatic ecology (Martini et al., 2021) has motivated recent studies to quantify copepod functional traits such as size, feeding mode, lipid content, or egg production in order to gain better insights into pelagic ecosystems functioning (Beaugrand, 2002; Benedetti et al., 2016; de Melo Júnior et al., 2021; Horne et al., 2016; Kinnard et al., 2011; Orenstein et al., 2022; Schmid et al., 2018; Schneider et al., 2017). Because carotenoid-based pigmentation significantly impacts copepod fitness, indicating a "good health" state, redness could be added to the list of key zooplankton traits routinely monitored.

For a sample of a few individuals, quantification of astaxanthin pigmentation can be done by classic HPLC measurements, while quantification at the individual

level can be done from zooplankton images using specific computer vision algorithms (Brüsin et al., 2016; Snoeijs & Häubner, 2014). Using automatic underwater cameras, pigmentation could also be inferred, at least by gray-level variations in black and white images (Vilgrain et al., 2021). At the community level, satellite imaging has been used to identify red patches in surface layers of arctic and subarctic marine ecosystems (Basedow et al., 2019). Using those technics, monitoring programs could derive such redness indices to obtain information on zooplankton quantity and quality, and hence on favorable foraging areas for predators such as whales, fish, and birds (Basedow et al., 2019; Berge et al., 2012; Fortune et al., 2020).

Copepods' carotenoid quantification has significant implications to estimate the transfer of antioxidants throughout food webs, from primary production to human consumers, and to evaluate the quality of fish stocks. Indeed, fish larvae exhibit a better coloration when they eat copepods fed with a diet enriched with carotenoids (Azani & Rasdi, 2021; Hynes et al., 2009), and the benefits of astaxanthin in fish for antioxidant and anti-inflammatory properties, egg quality, growth performance, immune system, lipid metabolism, or muscle pigmentation are unequivocal (Nakano & Wiegertjes, 2020). For example, in Inuit communities relying on a traditional diet for their food security, salmonid fish (*Arctic char*) are chosen for their color traditionally known to reflect their quality (Bolduc, 2021). Carotenoid-rich (fish) diets have indeed been proven beneficial for human health with therapeutic potential for Alzheimer, Parkinson, cardiovascular diseases, and cancer (Donoso et al., 2021), to the point that some companies began to harvest copepods to produce carotenoids-rich dietary supplements for humans (Dad'ová et al., 2020; Eysteinsson et al., 2018; Pedersen et al., 2014). Accumulation of carotenoid pigments functioning as powerful antioxidants through the food chain thus represents an ecosystem service (Sandifer & Sutton-Grier, 2014), and performing routine measurements of copepods' color could provide new information for a better monitoring of aquatic ecosystem health, also useful for human populations depending on them (Table 2).

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
No data were collected for this study. Qualitative and quantitative information from the literature and code (Vilgrain, 2023) are available from Zenodo: https://doi.org/10.5281/zenodo.7671742.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.