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1 **Storage of carotenoids in crustaceans as an adaptation to modulate immunopathology**
2 **and optimize immunological and life-history strategies**

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21 **Abstract**

22 Why do some invertebrates store so much carotenoids in their tissues? Storage of carotenoids
23 may not simply be passive and dependent on their environmental availability, as storage
24 variation exists at various taxonomic scales, including among individuals within species.
25 While the strong antioxidant and sometimes immune-stimulating properties of carotenoids
26 might be beneficial enough to cause the evolution of features improving their assimilation and
27 storage, they may also have fitness downsides explaining why massive carotenoid storage is
28 not universal. Here, we examine the functional and ecological implications of carotenoid
29 storage for the evolution of invertebrate innate immune defences, especially in crustaceans,
30 which massively store carotenoids for unclear reasons. We propose three testable hypotheses
31 about the role of carotenoid storage in immunological (resistance and tolerance) and life-
32 history strategies (with a focus on ageing), which may ultimately explain the storage of large
33 amounts of these pigments in a context of host-pathogen interactions.

34 **Introduction**

35 Carotenoids are natural lipid-soluble bioactive pigments, produced by photosynthetic
36 organisms (plants, bacteria, and algae) in which they absorb light for photosynthesis and
37 provide photoprotection.^[1] Apart from the rare exceptions involving symbiotic interactions
38 with microorganisms,^[2,3] animals cannot produce carotenoids themselves and must acquire
39 them from their diet.^[1,4] There are over 600 carotenoids known as xanthophylls and carotenes
40 when containing or not oxygen, respectively.^[4] Despite such a great diversity of carotenoids in
41 nature, only a few of them are absorbed and stored in animal tissues and fluids.^[5-7] This
42 suggests that they are either selectively accumulated by animals and/or metabolically
43 transformed for their storage.^[8] The amounts of carotenoids stored by animals vary across
44 taxa^[9,10] and within species,^[11] suggesting variability in their physiological importance in
45 addition to fluctuations in their environmental availability.

46 Because they are involved in many biological functions,^[4,5] carotenoids may have
47 strong implications in evolutionary biology. Their storage was suggested as indicative of
48 individual quality, such that carotenoid-colored traits could be sexually selected.^[12] They have
49 been found to have beneficial effects on survival, growth, and immunity, although this is still
50 strongly debated, especially in the bird literature from which most of the studies were
51 conducted.^[5,13-17] Non oxygenated carotenoids, such as beta-carotene can be important
52 precursors to vitamin A in many animals, whereas oxygenated ones, also called xanthophyll
53 carotenoids, such as astaxanthin and lutein, are not.^[18-20] Astaxanthin, mainly produced by
54 fungi and algae, is massively stored by aquatic animals.^[8] Specifically, most crustaceans
55 accumulate massive amounts of astaxanthin as circulating lipid droplets in the haemolymph
56 and in the eggs, and as esterified forms in their tissues.^[7, 21-23] This pigment, rarely found in
57 large amounts in vertebrates except for reports in salmonid fish^[8, 24-26], is essentially produced
58 from the metabolism of other carotenoids acquired in the diet^[27, 28] and is among the

59 carotenoids having the highest antioxidant potential.^[29] The precise reasons for such
60 substantial pigment accumulation in crustaceans remain unclear. However, its accumulation
61 has not been related so far to sexual signalling. Furthermore, while the involvement of
62 carotenoids as antioxidants in birds appears inconclusive (Koch et al. 2018), astaxanthin in
63 crustaceans, by contrast, may act as an important antioxidant (Ambati et al., 2014; Snoeijs and
64 Häubner, 2014; Schneider et al., 2016; de Carvalho and Caramujo, 2017; Weaver et al. 2018).
65 We propose that the strong antioxidant and the frequent immune-stimulating roles of
66 astaxanthin may limit the immunopathology cost associated with the immune response of
67 crustaceans.^[11, 30, 31]

68 Parasitism is an important source of stress for organisms. While the immune defences
69 protect from parasitic and pathogenic agents (here after pathogens), inflammatory responses
70 from the innate immune system also cause immunopathological disorders. Inflammation is a
71 phenomenon known from both vertebrates and invertebrates, corresponding to a fast but non-
72 specific response characterised by the delivery of fluids, cytotoxic chemicals and cells to
73 damaged and infected tissues, in order to fend off infectious agents and initiate tissue repair.
74 The cytotoxic chemicals released by the respiratory burst comprise highly reactive oxygen
75 species (ROS) and reactive nitrogen species (RNS) toxic to both pathogens and hosts' tissues,
76 leading to immunopathology.^[32] When damaged tissues are not fully repaired and
77 homeostasis is not fully restored, inflammation can further develop into a chronic condition,
78 with inevitable long-term debilitating consequences, such as increased rates of morbidity and
79 mortality at older age.^[32]

80 Organisms have evolved physiological processes that reduce the impact of
81 inflammatory injury, thus limiting the immunopathology costs. The integrated antioxidant
82 defence system controls the balance between the production and the detoxification of
83 cytotoxic free radicals, produced by basal metabolic activity and the immune response. This

84 system combines endogenous antioxidant enzymes, such as superoxide dismutase, catalase
85 and glutathione peroxidase, with exogenous (diet-acquired) antioxidants, such as vitamin E
86 (tocopherol), vitamin C (ascorbate), polyphenolic antioxidants, and carotenoids.^[33, 34] Among
87 these compounds, carotenoids may scavenge free radicals produced by immune activity
88 because of their long carbon skeleton containing conjugated double bonds and, in some cases,
89 oxygenated end rings.^[35-38] They also have the potential to interact with endogenous
90 antioxidant enzymes.^[31, 39, 40]

91 Here, we emphasize that the environmental availability of carotenoids and the ability
92 of organisms to assimilate and store these pigments, may impact the evolution of innate
93 immunity, by improving the defences against pathogens while reducing the fitness costs
94 associated with the inflammation response. We particularly focus on crustaceans for several
95 reasons, developed in the dedicated section below: their immunity relies on innate immune
96 systems alone, thus leading to immunopathology^[41]; they are known to accumulate naturally
97 large amounts of astaxanthin, a carotenoid with a particular high antioxidant ability^[29], which
98 is also frequently reported to enhance their immunity.^[31, 42-45]; and carotenoid storage in
99 crustaceans has not been involved so far in communication displays, which simplifies the
100 investigation of its functions. We first briefly review the mechanisms underlying the
101 invertebrate inflammatory response, with a special focus on the production of ROS and RNS.
102 After describing the integrated antioxidant defence system that includes dietary carotenoids,
103 we discuss the role of astaxanthin in modulating immunopathology, immunological strategies
104 (tolerance *versus* resistance) and ageing. Finally, we explore whether crustaceans could have
105 evolved the ability to store large amounts of astaxanthin in their tissues to maintain higher
106 levels of innate immune defences at lower immunopathological costs in response to local
107 pathogens.

108

109 **Innate immunity and immunopathology in invertebrates**

110 Invertebrate innate immunity is a costly system of protection that detects and eliminates a
111 wide variety of pathogens and unhealthy tissues. It comprises constitutive defences relying on
112 immune cells (haemocytes or coelomocytes) and several rapidly inducible enzymatic cascades
113 (e.g. the prophenoloxidase cascade) at the core of the inflammatory response.^[46] Upon
114 infection, recruited phagocytic immune cells increase their oxygen consumption during the
115 respiratory burst that leads to ROS production (Fig. 1), enhancing pathogen killing within
116 phagocytic cells and in the vicinity of extracellular bound pathogens.^[47] ROS are produced by
117 the activity of the membrane bound NADPH oxidase enzymatic complex, which catalyses the
118 production of NADP⁺ and cytotoxic superoxide anions (O₂⁻). Superoxide anions can then be
119 transformed into other damaging and unstable molecules, such as hydroxyl radicals OH[•], and
120 hydrogen peroxide H₂O₂ forming hypochlorous acid HOCl in reaction with chloride.^[48]
121 Recruited immune cells can also generate RNS, such as nitric oxide (NO).^[49, 50] In immune
122 cells, the synthesis of NO by inducible nitric oxide synthase, iNOS, has a broad anti-
123 pathogenic action (Fig. 1). NO can react with ROS yielding other highly reactive radicals
124 (Fig. 1): combined with hydrogen peroxide, it yields hydroxyl radicals,^[48, 51] combined with
125 superoxide anions, it yields oxidant and nitrating peroxynitrite radicals, ONOO⁻, that can
126 subsequently damage a large range of the cell molecules, inducing apoptotic or necrotic cell
127 death.^[48, 52]

128 A major response of invertebrate immunity accompanying the rapid aggregation of the
129 immune cells with each other and with the pathogens is the melanisation reaction catalysed by
130 phenoloxidase enzymes (PO), which heal wounds through clotting and clear invading
131 pathogens through nodulation or encapsulation.^[53] POs are present as inactive proenzymes
132 (prophenoloxidase or proPO) in immune cells. They are released and converted into their
133 active form in the haemolymph upon wounding or infection. In crustaceans, infection may

134 further generate the cleavage of a fragment of the hemocyanin, a respiratory protein, which
135 also has PO activity.^[54] Melanin synthesis is accompanied by the production of ROS and
136 RNS, helping to kill intruders.^[50, 53] However, excessive PO cascade activity can
137 subsequently damage the host's own tissues and organs (Fig. 2).^[55, 56] Immunopathological
138 effects upon encapsulation and clotting are likely to be frequent in invertebrates since the
139 occurrence and abundance of wounding can be extremely high.^[57-59] Repeated wound clotting
140 and multiple melanisation reactions in response to repeated infections may be then harmful, as
141 it exposes the host organs to cumulative cytotoxic radicals in the open circulating system.

142

143 **Carotenoids as part of the integrated antioxidant defence system**

144 The accumulation of cytotoxic ROS and RNS is limited by the detoxifying activity of the
145 integrated antioxidant system, including endogenous enzymes and non-enzymatic exogenous
146 compounds.^[33, 60] Three major antioxidant enzymes detoxify free radicals (see Fig. 1).
147 Superoxide dismutase transforms highly reactive superoxide anions O_2^- into hydrogen
148 peroxide. Then catalase converts hydrogen peroxide into water. Hydrogen peroxide
149 detoxification can also be catalysed by peroxidase when ROS are associated with substrates
150 that can be oxidised.^[33, 61] In addition to these three major enzymes, crustaceans possess
151 peroxinectins, proteins with multiple roles in immune defences, including peroxidase activity.
152 Upon immune challenge, peroxinectins are released by haemocytes after the activation of the
153 proPO cascade, bound to extracellular superoxide dismutase, thus helping to detoxify the
154 hydrogen peroxide produced by superoxide dismutase activity.^[62, 63] However, peroxinectins
155 seem to have lower peroxidase activity than catalase upon immune challenge.^[31]

156 Non-enzymatic exogenous compounds with antioxidant properties are acquired
157 through dietary intake and presumably help antioxidant enzymes to keep the oxidative burden
158 under control. These compounds act either as hydrogen donors (chemical traps), or as free

159 radical scavengers (physical traps).^[33, 60, 64] Non-enzymatic antioxidants are divided into
160 hydrophilic compounds, present in aqueous cellular compartments (e.g. vitamin C,
161 glutathione), and lipophilic compounds (e.g. carotenoids), which can be included in cell
162 membranes (Fig. 1) and associated with lipoproteins.^[33, 60] All these exogenous antioxidant
163 compounds being acquired from the diet, their environmental availability can be limited and
164 limiting. Hence, this environmental availability, as well as the ability to assimilate these
165 antioxidant compounds, will be a crucial parameter influencing the potential synergy with
166 endogenous antioxidant enzymes and in the general antioxidant capacity. Among these
167 compounds, carotenoids are powerful antioxidants^[35, 36] and relatively abundant in natural
168 food sources.^[65] Unlike other dietary antioxidants, they can be stored in large amounts in
169 animal tissues.^[7, 66] Hence, carotenoids potentially play a major role in the regulation of
170 immunopathology, and therefore in the modulation and the evolution of immune defences.

171

172 **Crustaceans as systems for studying physiological and evolutionary implications of** 173 **carotenoid storage**

174 Along with their biological proprieties, carotenoids are involved in several functions
175 including in communication displays in birds, amphibians, and reptiles. Carotenoid-based
176 coloration of the skin and plumage has been found to be an honest secondary signal of
177 resistance to oxidative stress, body condition, and immunocompetence.^[5, 25] Several
178 hypotheses on the molecular mechanisms underlying signal honesty have been formulated,
179 including the allocation trade-offs and shared molecular pathways, leading to specific
180 expected relationships between stored amounts of carotenoids and other physiological
181 parameters.^[15, 67, 68] Nevertheless, it has been suggested that stored carotenoids may be minor
182 antioxidants for birds, preferentially used for sexual signalling, while other non-pigmentary
183 antioxidants (e.g. melatonin) may be used primarily for detoxification during immune

184 activity.^[69] In these bird examples, carotenoid-based coloration might have been under strong
185 selection, leading to diversion of these pigments for sexual signalling. Therefore, taxa that do
186 not use these pigments for communication displays would be more straightforward to study
187 the role of carotenoids in alleviating oxidative stress in immunopathology, avoiding
188 interferences with signalling.

189 In invertebrates, aquatic crustaceans are among the greater animal reservoirs of
190 carotenoids by accumulating mostly astaxanthin in their tissues, cuticle, haemolymph, and
191 eggs.^[7, 9, 22, 28] They can obtain this pigment in their diet by feeding on various algae and
192 microbes that are producing them.^[8-10] However, the most common carotenoids contained in
193 their food are typically beta-carotene, zeaxanthin, lutein, and other less abundant carotenoids
194 that crustaceans are oxidizing to produce the astaxanthin they accumulate.^[27, 28] They can also
195 directly obtain astaxanthin by consuming animals that have already performed the
196 bioconversion of other carotenoids into astaxanthin and stored it. Astaxanthin has the highest
197 antioxidant activity among natural carotenoids: it is 10 times more effective than β -carotene
198 and over 100 times more effective than vitamin E.^[70-72] However, the presence of salt lowers
199 the relative oxidant activity of astaxanthin.^[73] Assuming that animals store astaxanthin mainly
200 for its antioxidant potential, it may not be surprising that some marine organisms
201 (crustaceans, but also some fish, see the introduction) store them in large amounts to
202 compensate for its reduced antioxidant activity in salty water, although this needs to be
203 formally tested. Astaxanthin also has unique other biochemical properties making it able to
204 form chelate complexes with metals that could otherwise impair mitochondrial function.^[73] Its
205 ability to be esterified allows a better stabilization of the free form and prevents its
206 aggregation that would otherwise favour pro-oxidant effects.^[29] The latter phenomenon could
207 be further prevented by the ability of the pigment to form proton loss neutral radicals.^[29]
208 Together, these properties likely enable the accumulation of large amounts of astaxanthin

209 with limited negative effects, making it an efficient protective agent against environmental
210 stressors.

211 The natural storage of large quantities of astaxanthin seems to be the healthy/normal
212 situation for crustaceans, whereas the food depletion in carotenoids seems to be unusual; the
213 astaxanthin storage rate depending on the pigment nature and availability.^[28] In freshwater
214 and marine crustaceans, the storage of astaxanthin was associated to numerous beneficial
215 physiological effects, including immunity^[74-76] and antioxidant defences^[77, 78] but has never
216 been reported to be involved to sexual signalling, either directly or indirectly. By contrast
217 with vertebrate and insect immunity, crustacean immunity relies strongly on hemocytes, the
218 cytotoxic activity of the proPO cascade and on the production of ROS/RNS. The large panel
219 of antimicrobial peptides described in insects, allowing them to efficiently control microbial
220 infections without generating self-damage, has no equivalent, so far, in crustaceans.^[79]
221 Crustacean immunity is therefore strongly dependent on self-reactive processes to combat
222 infection by pathogens. Furthermore, crustaceans have an open circulatory system frequently
223 exposed to microbes often in high concentrations in their natural habitat.^[80-82] All these
224 elements suggest that storage of carotenoids has a central role in the physiology of
225 crustaceans, which make then an important model system to study the role of carotenoids in
226 the detoxification of the immune response.

227

228 **Hypothesis 1: carotenoids modulate immunological strategies of crustaceans by limiting** 229 **immunopathology**

230 Animals may survive infection through two non-mutually exclusive immunological strategies:
231 resistance and tolerance.^[83, 84] Resistance reduces pathogen burden by the action of the host's
232 immune system, but leads to significant immunopathology, and hence to fitness costs (see
233 above). Alternatively, the host may restrain its immune response and tolerate a certain

234 pathogen burden, limiting the immunopathology associated with the immune response. These
235 two immunological strategies could be closely linked, as limiting immunopathology
236 (tolerance strategy) may allow the host's immune system to be more efficient at eliminating
237 pathogens (resistance strategy).^[85] Therefore, any mechanism limiting immunopathology is
238 expected to have profound implications for the evolution of tolerance and/or resistance,^[32, 86]
239 and for host-pathogen interactions.^[85]

240 Here, we propose that the immune-stimulant and antioxidant activities of carotenoids
241 could influence the crustacean's resistance and tolerance immunological strategies, by
242 enhancing immune response efficiency while limiting immunopathology. This would translate
243 into a very efficient and lower-cost immune function resulting from the storage of the storage
244 of large amounts of astaxanthin.^[30, 31] Carotenoids could improve resistance as they were
245 reported to stimulate immune defences of vertebrate animals^[e.g. 5, 15, 16, 87], although this is not
246 always the case.^[14, 37, 38, 88] In marine and freshwater crustaceans, immunity is frequently
247 improved by dietary carotenoids.^[31, 42-45] For instance, in the amphipod crustacean *Gammarus*
248 *pulex*, experimental dietary supplementation with carotenoids enhanced immune activity and
249 increased resistance to microbial infection (Fig. 2).^[30] Dietary supplementation with
250 carotenoids increases phenoloxidase activity and total haemocyte count in the giant
251 freshwater prawn *Macrobrachium rosenbergii*.^[89] Furthermore, the injection of astaxanthin in
252 the same species increases total haemocyte count and survival when exposed to the
253 pathogenic bacterium *Lactococcus garvieae*.^[90] Likewise, a dietary supplementation with
254 astaxanthin increased the total haemocyte count of the marine whiteleg shrimp *Litopenaeus*
255 *vannamei* juveniles.^[43] Similarly, a yeast-rich diet containing carotenoids increased total
256 haemocyte count and phenoloxidase activity in the marine Indian prawn *Fenneropenaeus*
257 *indicus*.^[44] The mechanisms through which carotenoids directly or indirectly enhanced basal
258 levels of immune effectors are still unknown. Nevertheless, recent findings suggest that

259 astaxanthin exhibits antimicrobial activity against a large range of microbes *in vitro* through
260 yet unknown mechanisms.^[91, 92] Storage of this pigment could therefore provide a direct
261 advantage upon microbial infection.

262 Carotenoids could also improve tolerance in several ways. They may prevent oxidative
263 stress-mediated damage resulting from the immune response by scavenging cytotoxic free
264 radicals (Fig. 1),^[35] helping in their detoxification,^[36] hence limiting the immunopathology
265 cost (Fig. 3). This might be particularly important in crustaceans whose immunity strongly
266 relies on auto-reactive immune responses in an open circulating system where the organs
267 bathe in the haemolymph. These processes are suspected to occur in carotenoid-supplemented
268 *G. pulex*, which do not suffer from additional survival costs compared to control gammarids,
269 despite exhibiting stronger immune activity and resistance to microbes.^[30] From a functional
270 perspective, improved tolerance mediated by carotenoids in *G. pulex* might result from the
271 take-over of the activity of the antioxidant enzyme superoxide dismutase in the detoxification
272 of the superoxide anions of the innate immune response, while the activity of the other
273 antioxidant enzyme catalase is promoted.^[31] Nevertheless, further studies are needed to better
274 understand the functional role of carotenoids in the detoxification process in association with
275 endogenous antioxidant enzymes.

276 Carotenoid-mediated tolerance may also result from interactions between the pigments
277 and the regulation of the host's metabolism.^[19, 93] Infections can have dramatic metabolic
278 consequences, characterised by the loss of energetic reserves. This results in elevated
279 circulation of glucose, amino acids and fatty acids, which can cause morbidity and death.^[94, 95]
280 Carotenoids, especially astaxanthin, have hypolipidemic and hypoglycemic effects in rodents
281 after supplementation.^[19, 96] Moreover, there is evidence that astaxanthin can interact with
282 nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) superfamily,
283 which regulates lipid and glucose metabolism.^[97] It is therefore possible that astaxanthin may

284 prevent metabolism dysregulation upon infection through interaction with PPARs. If so,
285 carotenoids may not only improve tolerance by preventing wasting of metabolic reserves, but
286 also impact resource trade-offs involving the immune function upon immune challenge.

287 Beyond the functional characterisation of the interactions of carotenoids with the host
288 physiology, testing the above hypothesis relies on determining the phenotypic consequences
289 of the dietary supplementation with the pigments. Especially, under the above hypothesis, the
290 supplementation of the food of crustaceans with carotenoids is expected to improve the
291 survival and reproductive success of hosts exposed to infectious or non-infectious immune
292 challenges if the pigments enhance immunity or tolerance, respectively.

293

294 **Hypothesis 2: carotenoids modulate ageing in crustaceans by limiting immunopathology**

295 Ageing is the progressive deterioration of physiological functions with age, leading to age-
296 dependent decline in fecundity and increased mortality.^[98] Several non-mutually exclusive
297 hypotheses have been put forward to explain ageing, and many involve cumulative oxidative
298 damage by biological processes.^[99, 100] Mechanisms such as antagonistic pleiotropy, when the
299 expression of genes is beneficial early in life but becomes adverse at older age,^[101, 102] and
300 damage by ROS and RNS, all contribute to ageing.^[103, 104] Ageing and inflammation-mediated
301 immunopathology are intrinsically linked, as ageing is characterised by a prolonged pro-
302 inflammatory status as a result of an imbalance between pro- and anti-inflammatory status. As
303 stated above, ROS and RNS accumulation is a prominent theory of ageing,^[103, 104] and their
304 regulation in the host when interacting with pathogens appears critical in the ageing
305 process.^[32, 56] It has been proposed that early-life inflammation could have long-term or
306 delayed pathological costs, yielding increased morbidity and mortality at older age.^[105, 106] In
307 line with this, a non-pathogenic immune response experimentally elicited in early life in the
308 mealworm beetle, *T. molitor*, reduces adult life span.^[32] In this insect, activation of the

309 prophenoloxidase cascade and the release of ROS and RNS by haemocytes in response to
310 non-self have an immunopathological effect on Malpighian tubules (insect ‘kidneys’), leading
311 to a lower secretion efficiency. Their function is impaired on the long term,^[55] causing ageing
312 acceleration^[56] and shortening lifetime reproductive success. Preventing immunopathology
313 early in life may reduce ageing acceleration and therefore improve lifetime reproductive
314 success. The enzymatic and non-enzymatic antioxidant scavengers, together with a variety of
315 molecular repair processes, can help to limit immunopathology, and therefore ageing.

316 Given all these elements, we hypothesise that, in the context of ageing, fitness benefits
317 from the storage of antioxidant carotenoids in crustaceans, especially astaxanthin for
318 crustaceans, in dealing with inflammation consequences such as immunopathology, should be
319 greater at early age than at older age. Given its biological properties mentioned earlier, this
320 would be particularly the case for the storage of astaxanthin after its stabilisation as esterified
321 forms for storage in crustacean tissues.^[7, 8, 24] This might also be particularly relevant in
322 animals whose immunity relies almost exclusively on inflammatory responses, like again in
323 crustaceans. After infection at early stages, carotenoids are expected to help keep the balance
324 between pro- and anti-inflammatory status under control for a longer period of time,
325 preventing ageing acceleration and preserving lifetime reproductive success (Fig. 3). Hence,
326 maternal transfer of carotenoids into the eggs might be highly advantageous to prevent ageing
327 acceleration in offspring in septic environments.^[107, 108] In the particular case of crustaceans,
328 we therefore predict that stored astaxanthin will have a greater positive effect on lifespan and
329 early reproductive success early in life. In line with this, small gammarid crustaceans, that are
330 the youngest, store higher quantities of carotenoids in their haemolymph than larger
331 individuals, which are the oldest.^[30] Whether this age-related pattern of carotenoid storage is
332 adaptive in the context of ageing is not yet known. Its consequences on lifetime reproductive
333 success and ageing have to be tested.

334 In crustaceans, ageing is also likely further prevented by the unique properties of the
335 astaxanthin they accumulate. In addition of its high antioxidant activity, astaxanthin is able to
336 form chelate complexes with metal ions, such as Zn^{2+} , which is known to inhibits the
337 mitochondrial complex I (NADH: ubiquinone oxidoreductase)^[109], that is central for energy
338 transduction and whose dysfunction is implicated in neurodegenerative and muscular diseases
339 and in ageing.^[29, 73]

340

341 **Hypothesis 3: carotenoids storage is adaptive to fight pathogens**

342 Some organisms, including crustaceans, have evolved carotenoproteins (i.e. carotenoids in
343 association with proteins)^[76, 110] that allow the storage of large amounts of carotenoids in their
344 tissues for yet unknown reasons, and its involvement in sexual selection has never been
345 reported so far (see the above section presenting crustaceans as relevant models systems to
346 address the proposed hypotheses). Most crustaceans feed on decaying organic matter,
347 probably full of obligate and opportunistic pathogens.^[111] Crustaceans also often live in
348 microbes-rich aquatic environments that almost permanently expose their open circulatory
349 system to microbial infections.^[80-82] From a host-pathogen interaction perspective, given the
350 beneficial roles of carotenoids, especially astaxanthin, in the regulation of the immune
351 function and associated immunopathology,^[31, 43, 75] we propose that, in these organisms, the
352 storage of large amounts of carotenoids^[30] and the maternal transfer of carotenoids^[7, 28] could
353 have evolved to deal with pathogen attacks, using the more efficient inflammatory response
354 provided by carotenoid enrichment. On the one hand, selection imposed by pathogens should
355 favour resistance, giving rise to a more efficient immune response and higher levels of
356 immune defences.^[112, 113] On the other hand, since antioxidant defences are crucial to deal
357 with the oxidative stress associated with the immune response and with the pathogens
358 themselves,^[114] improved tolerance should be favoured simultaneously, yielding more

359 efficient antioxidant defences through increased antioxidant enzyme production for instance.
360 Hence, interactions with pathogens should increase *broad sense* immune costs, i.e. direct
361 costs due to resource consumption by the immune response, and indirect costs resulting from
362 immunopathology and the use of enzymatic antioxidant defences.^[55, 114-116] The storage of
363 immune-stimulant and antioxidant carotenoids should alleviate all these operating costs,
364 providing crustaceans with a very efficient and lower-cost immune function.^[30, 31] In this
365 context, we hypothesise that there should be detectable correlations between the pathogen
366 prevalence and the quantities of stored carotenoids among crustacean populations. Crustacean
367 populations exhibiting higher pathogen prevalence, i.e. crustacean populations that are
368 exposed to more frequent pathogen attacks, should evolve greater capacity of carotenoid
369 storage in the tissues and/or in the haemolymph. While, on the one hand, such a relationship
370 is likely driven by the potential benefits of storing large amounts of carotenoids under high
371 pathogen pressure, on the other hand, it might also be driven by potential costs associated to
372 carotenoids storage under low pathogen pressure.

373 Indeed, the above elements indicate that carotenoid storage is highly advantageous.
374 However, natural variation in the amounts of stored carotenoids persist at each taxonomic
375 level in animals, among phyla,^[9] species,^[10] and populations within species.^[11] This variation
376 could reveal local differences in the environmental availability of carotenoids. It could also
377 result from fitness costs associated with carotenoid storage. Under the hypothesis that
378 carotenoid storage evolved to support the immune system in fighting pathogens, the
379 expression of its costs should be maximal in the absence of pathogens. Moreover, the fact that
380 carotenoid storage occurs despite bearing fitness costs would strongly support its adaptive
381 nature. While detrimental effects of carotenoids have been suggested,^[12] the potential fitness
382 costs associated with their storage remain poorly investigated even in animals storing
383 naturally these pigments. In copepod crustaceans, carotenoid reserves decrease the infection

384 risk but increase the predation risk by making them conspicuous to predators.^[117-119] In birds,
385 negative effects of high doses of carotenoids were reported on skeletal muscles and
386 reproduction, mainly under relatively non-stressful conditions, suggesting context-dependent
387 effects of carotenoids.^[120, 121] Similarly, in the mealworm beetle, *T. molitor*, carotenoid
388 supplementation provided larvae with a survival benefit under immune challenge, while it
389 was associated with a survival reduction and prolonged larval development without immune
390 challenge.^[122] Furthermore, at high concentrations, carotenoids may potentially reduce the
391 immune response efficiency, through their high capacity to scavenge the free radicals released
392 by the immune system, whose function is to kill pathogens. In some invertebrates, carotenoids
393 may also potentially down-regulate the immune response by interfering with the cellular NO
394 signalling, known as a major regulator of the insect immune response.^[123-126] Indeed, when
395 NO stimulates both cellular and humoral immunity of insects, the strong antioxidant power of
396 carotenoids may scavenge a fraction of circulating NO, thus down-regulating basal immune
397 activity. In addition, certain carotenoids, such as astaxanthin, were reported to inhibit the
398 enzymatic activity of the nitric oxide synthase responsible for NO production from L-arginin
399 (Fig. 1), which activity is normally increased upon immune challenge.^[19, 124] Such a general
400 immune depressive effect of carotenoids was recently evidenced in *T. molitor*, increasing the
401 susceptibility to entomopathogenic infections;^[122] It contrasts available evidence showing that
402 carotenoids improve the immune response success in crustaceans.^[30, 31, 43-45, 89, 90] However,
403 since crustaceans have evolved specialized features to store large amounts of carotenoids in
404 their tissues in contrast to insects,^[76] these pigments might be of special importance in their
405 physiology, including in immunity. This suggests that dietary carotenoids may be challenging
406 for biological systems, at least for those that did not evolved specialized features to store
407 them, and that beneficial and detrimental effects resulting from carotenoid diet
408 supplementation might be host specific and context-dependent. Carotenoid storage might also

409 have undiscovered costs, leading to spatial and temporal natural variation within and among
410 invertebrate populations. It is therefore necessary to study the origins of natural variation in
411 carotenoid content to know whether it is adaptive and to understand the conditions of
412 carotenoid storage evolution.

413 Another important element for the storage of carotenoids to be adaptive is that its
414 expression should be genetically or epigenetically encoded and therefore heritable so that
415 natural selection can act on it. So far, no available studies have investigated whether the
416 capacity to store carotenoids is heritable. Under the hypothesis that carotenoids storage is
417 adaptive to fight pathogens, genetic variance and heritability for this trait might therefore be
418 expected. Ample additive genetic variance and heritability have been found for components
419 of the innate immune response in invertebrates.^[127] Investigating the genetic basis of
420 carotenoid storage by measuring its heritability and estimating potential genetic correlations
421 with components of the innate immune response as well as other life-history traits appears of
422 primary importance. In particular, in addition to providing information on how much selection
423 could act on the expression of carotenoid storage, this approach may also reveal to what
424 extent it is associated to the expression of the innate immune response to pathogens.

425

426 **Conclusions and perspectives**

427 Despite its benefits against infections, the inflammatory immune response, through releasing
428 oxidative cytotoxic molecules, has inevitable immunopathology costs, which decrease
429 lifetime reproductive success and longevity.^[32] Endogenous antioxidant enzymes coupled
430 with exogenous antioxidant compounds, including dietary carotenoids, help to limit
431 immunopathology.^[105] Carotenoids are powerful antioxidants^[35, 36] able to limit the oxidative-
432 mediated self-harm resulting from the immune response. Hence in addition to directly or
433 indirectly stimulate immunity in some organisms^[30, 42- 45, 87, 8 2, 90], carotenoids may limit the

434 persistence of a pro-inflammatory status causing age-associated morbidity and death.^[103, 104]
435 Furthermore, carotenoids such as astaxanthin, may prevent metabolic wasting upon infection
436 by influencing the host's metabolism regulation.^[96,97, 128] Unlike other dietary antioxidants,
437 carotenoids can be stored in animal tissues and, for yet unknown reasons, aquatic crustaceans
438 exhibit particularly impressive capacities to store astaxanthin compared to other taxa.^[7, 9, 66]
439 From these observations, we infer that greater carotenoid storage in crustaceans could be
440 adaptive against pathogens.

441 To examine this possibility, we propose three testable hypotheses:

- 442 (1) As astaxanthin is antioxidant and immune-stimulant, the evolution of the crustacean
443 immune function and enzymatic antioxidant defence system may have occurred in a particular
444 context, because of their capacity to store large amounts of carotenoids. Large astaxanthin
445 storage should therefore promote both resistance and tolerance to infection, providing
446 crustaceans with a highly efficient immune response, but at low immunopathological costs.
- 447 (2) As inflammation-associated immunopathology is closely linked to ageing, storage of
448 carotenoids should prevent ageing acceleration resulting from early-life infection and
449 therefore enhance lifetime reproductive success. In this context, maternal transfer of
450 carotenoids into the eggs and the storage of these pigments early in life should be
451 advantageous in a septic environment.
- 452 (3) By promoting resistance and tolerance, carotenoids may interfere in host-pathogen
453 interactions, which would lead to correlations between these pigments storage and the
454 pathogen prevalence among crustacean populations. Therefore, high pathogen pressure should
455 favour higher capacity to store carotenoids.

456 These above roles of carotenoid storage might be particularly important in aquatic
457 crustaceans, which live in microbes-rich environments constantly challenging their immune
458 system, compare to other animals^[80-82], at least for two main potential reasons. First, the

459 crustacean immune response strongly relies on hemocytes and their cytotoxic activity
460 resulting from the prophenoloxidase system and ROS/RNS production, and marginally on the
461 production of non self-reactive immune effectors such as the insect antimicrobial peptides and
462 the vertebrate antibodies of the acquired immune system.^[79] Second, aquatic crustaceans
463 have relatively large access to carotenoids in their habitat.^[8-10] One may speculate that such a
464 large carotenoid availability may have prevented the evolution of immune innovations
465 allowing a reduced use of ROS/RNS, like in insects.

466

467 **Author's contributions**

468 Y.M. conceived the ideas. A.B., J.M., and Y.M. performed the literature search and led the
469 writing of the manuscript.

470

471 **Competing interests**

472 We have no competing interests.

473

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477

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480

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657

658 **Figure legends**

659 **Figure 1.** Diagrammatic representation of the respiratory burst during phagocytosis by
660 immune cells and the possible action of carotenoids (astaxanthin). Foreign objects are
661 phagocytosed and encapsulated within a phagosome. NADPH oxidase is activated and
662 accepts electrons from NADPH to reduce O_2 to $O_2^{\cdot-}$ (superoxide anion). This anion could
663 interact with nitric oxide (NO), produced by the inducible nitric oxide synthase (iNOS) from
664 L-arginine to generate peroxynitrite ($ONOO^{\cdot-}$). Alternatively, $O_2^{\cdot-}$ could form hydrogen
665 peroxide (H_2O_2) by the superoxide dismutase (SOD) activity, which could be used by the
666 catalase or other peroxidases (POD) in a process of detoxification to produce water (H_2O) and
667 oxygen (O_2). Alternatively, the myeloperoxidase (MPO) uses H_2O_2 in the presence of chloride
668 (Cl^-), to generate hypochlorous acid (HOCl). H_2O_2 could also be used to produce again $O_2^{\cdot-}$
669 through Fenton's reaction or Haber-Weiss reaction. Carotenoids such as astaxanthin (ASTX;
670 orange molecules) neutralize free radicals by scavenging singlet oxygen anions on both side
671 of the cellular membrane. It could also inhibit the iNOS activity, thus limiting the production
672 of NO. Explosive stars illustrate the cytotoxicity of the molecule inside.

673

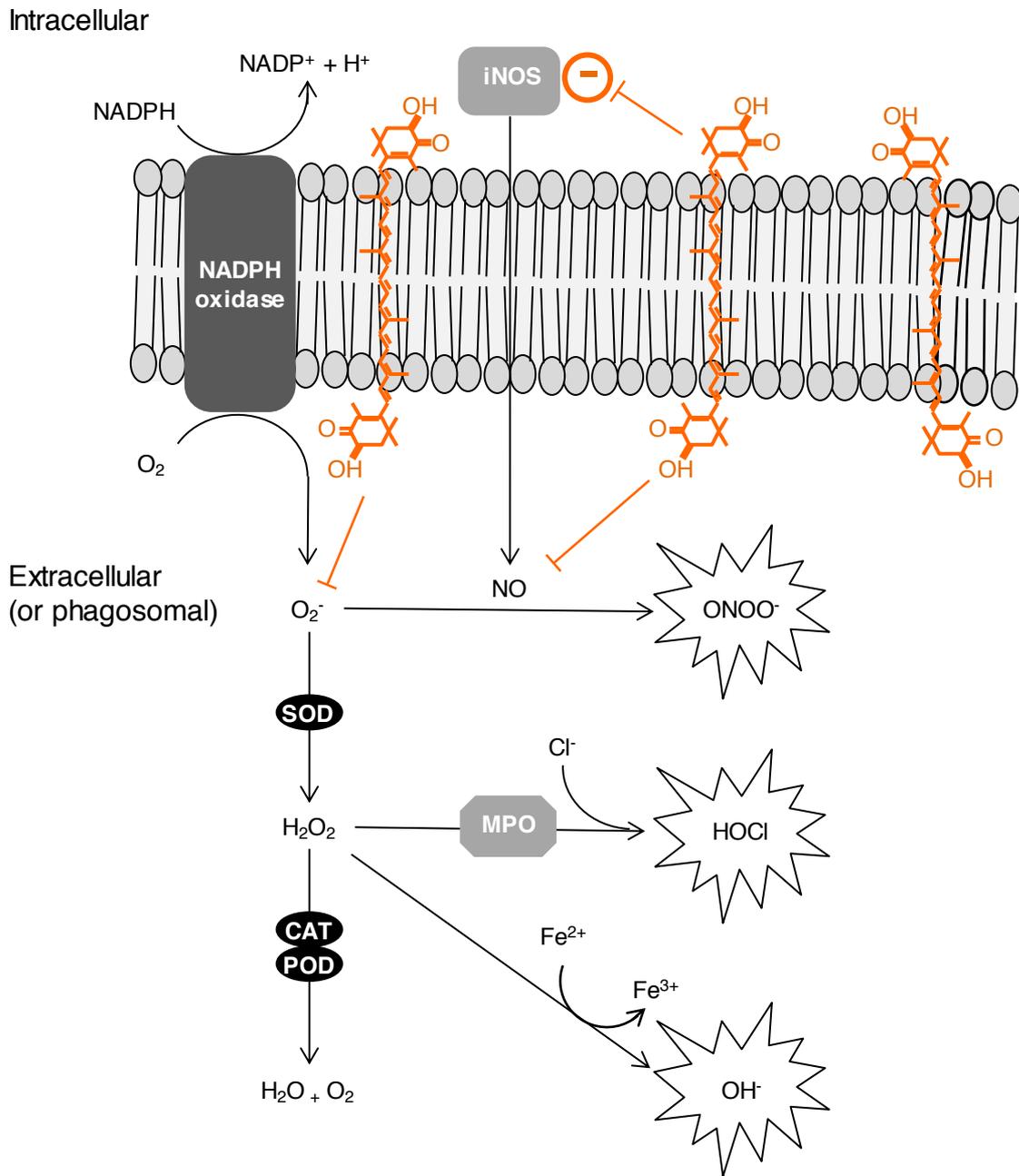
674 **Figure 2.** Induction of the immune response (phagocytosis and prophenoloxidase cascade)
675 after pathogen infection or wounding in crustaceans (plain grey arrows), and consequences on
676 host homeostasis (plain and dashed arrows). The immune response can successfully clear
677 infection by pathogens and repair wounds, leading back to homeostasis (plain grey arrow).
678 Alternatively, pathogen clearance and wound healing may not be entirely successful, leading
679 to increased self-harm, chronic inflammation and immunological oxidative stress (dashed
680 arrows).

681

682 **Figure 3.** Schematic view of the effects of the immune response on resistance to pathogens
683 and immunopathology (black arrows) and the hypothetical action of dietary carotenoids in this
684 system (orange arrows). Plus and minus signs refer to positive and negative effects on the
685 arrow targets. The immune response helps to control the growth of pathogens entering the
686 host, which is beneficial for host survival to pathogens. However, the immune response
687 induces the production of ROS and RNS, which have negative impacts on host survival by
688 inducing immunopathology and age-related diseases. Carotenoids might reduce the negative
689 effects of ROS and RNS by scavenging free radicals, or by stimulating enzymes in the
690 endogenous antioxidant system. Large filled orange arrows indicate the potential indirect
691 consequence of increasing the strength of the immune response, leading to better resistance to
692 pathogens. The large open dashed orange arrow with a plus sign between carotenoids and the
693 immune response indicates the potential direct stimulatory power of carotenoids on the
694 immune response. Thin dashed orange arrows indicate the carotenoid-mediated reduction of
695 the negative effects of ROS and RNS that will also improve host survival by decreasing the
696 probability of immunopathology and age-related diseases.

697

698 **Figure 1.**

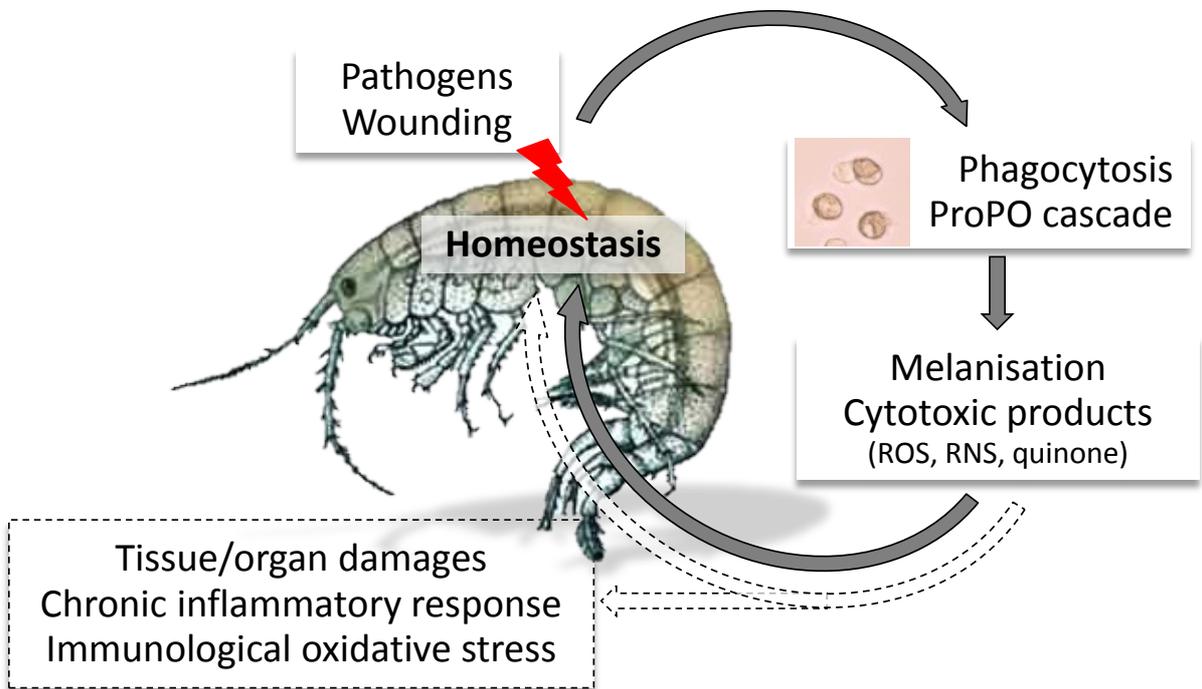


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700

701 **Figure 2.**

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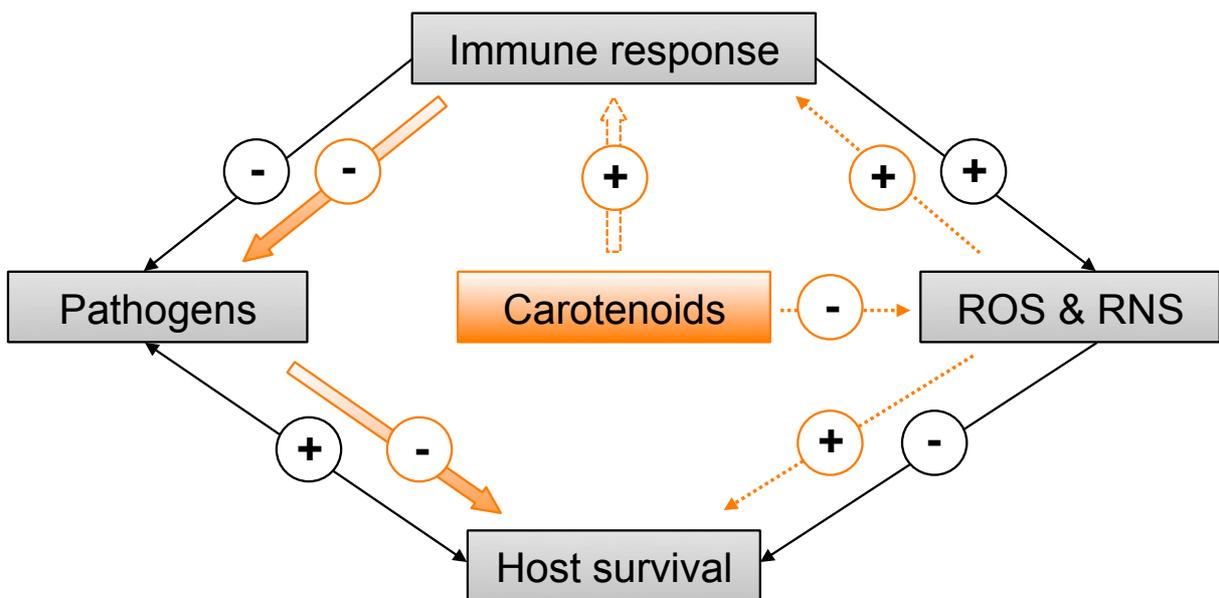


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704

705 **Figure 3.**

706



707