

Sex-specific effects of carotenoid intake on the immunological response to allografts in guppies (*Poecilia reticulata*)

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Rarely are the evolutionary origins of mate preferences known, but, recently, the preference of female guppies (*Poecilia reticulata*) for males with carotenoid-based sexual coloration has been linked to a sensory bias that may have originally evolved for detecting carotenoid-rich fruits. If carotenoids enhance the immune systems of these fishes, as has been suggested for other species, this could explain the origin of the attraction to orange fruits as well as the maintenance of the female preference for orange males. We used the classic immunological technique of tissue grafting to assay a component of the immune response of guppies raised on two different dietary levels of carotenoids. Individual scales were transplanted between pairs of unrelated fishes, creating reciprocal allografts. Transplanted scales were scored on a six-point rejection scale every day for 10 days. Five days later, the same pairs of fishes received a second set of allografts and were scored again. Compared with low-carotenoid-diet males, high-carotenoid-diet males mounted a significantly stronger rejection response to the second allograft but not to the first allograft. High-carotenoid-diet females, however, showed no improvement in graft rejection compared with low-carotenoid-diet females. To our knowledge, this is the first experimental evidence for sex-specific effects of carotenoid consumption on the immune system of a species with carotenoid-based sexual coloration. These results are consistent with the hypothesis that the mate preference for carotenoid coloration is maintained by the benefits to females of choosing healthy mates, but they cast doubt on the idea that the benefits of carotenoid consumption, *per se*, could account for the origin of the preference. The sex-specificity of carotenoid effects on allograft rejection in guppies provides indirect support for the general hypothesis that males pay an immunological cost for sexual ornamentation.

Keywords: carotenoid coloration; mate preference; immune response; allograft rejection; *Poecilia reticulata*

1. INTRODUCTION

Because of their dietary origin (Fox 1979; Goodwin 1984; Schiedt 1989), carotenoid-based colours may serve as direct signals of foraging ability and as indirect signals of vigour and health (Endler 1980), which could account for their prevalence in courtship and competitive displays (Kodric-Brown & Brown 1984; Andersson 1994). However, the use of carotenoids in animal communication may also be related to immune-system-enhancing effects of these pigments (Lozano 1994; Olson & Owens 1998; Møller *et al.* 2000). High levels of carotenoids in the integument may signal not only past good health but also the potential to respond to future health challenges (Lozano 1994; but see Hill 1999). This hypothesis has received support from studies correlating measures of immune-system function with the amounts of carotenoids circulating in the blood or deposited in the integument of species with carotenoid-based sexual coloration (reviewed in Møller *et al.* 2000). But clearly, to demonstrate a causal link between carotenoids and the immune system, it is

necessary to manipulate carotenoid levels experimentally and measure the effects on the immune system (or vice versa). Unfortunately, virtually all such experiments have been carried out on species that lack carotenoid-based sexual coloration (Hill 1999; but see Blount *et al.* 2003; Faivre *et al.* 2003).

We tested for effects of dietary carotenoids on the allograft-rejection response of male and female guppies (*Poecilia reticulata*). Guppies are small poeciliid fishes native to the tropical rainforest streams of Trinidad and nearby regions (Endler 1978). Males alone display orange carotenoid-containing spots, the size and chroma of which influence their sexual attractiveness (Kodric-Brown 1989; Houde 1997; Grether 2000). The primary source of carotenoids for guppies in nature is attached unicellular algae, the availability of which (in undisturbed streams) is largely a function of stream order (Grether *et al.* 2001). High-order streams tend to make larger gaps in the forest, receive more photosynthetically active light and contain larger standing crops of algae, but not higher densities of guppies, than low-order streams (Grether *et al.* 2001). Consequently, guppy populations in the upper reaches of a drainage basin tend to experience greater carotenoid limitation than those in the lower reaches (Grether *et al.* 1999).

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The preference of female guppies for males with large chromatic orange spots may have arisen as a pleiotropic effect of a sensory bias that originally evolved in the context of food detection (Grether *et al.* 2002; Rodd *et al.* 2002). In Trinidad, guppies are quick to find and consume carotenoid-rich orange-coloured fruits, which occasionally fall into the streams from the forest canopy (Rodd *et al.* 2002). In field and laboratory experiments, both sexes approach and nibble orange painted discs in preference to discs of other colours (Rodd *et al.* 2002), and this colour preference appears even in individuals with no prior experience of orange food (G. F. Grether, G. R. Kolluru and F. H. Rodd, unpublished data). Across populations, the degree of attraction to orange discs explains most of the genetic variation in the strength of the female preference for carotenoid coloration in males (Rodd *et al.* 2002). This suggests that attraction to orange objects and the mate preference for carotenoid coloration are genetically linked, and that selection on one of these traits could cause changes in the other as a correlated response. It seems likely that the mate preference arose as a side effect of the foraging preference rather than vice versa (Rodd *et al.* 2002). Once male coloration evolved to exploit this 'sensory trap' (Christy 1995), the mate preference could have been maintained, in part, by the benefits to females of mating with healthy high-carotenoid males. Thus, any benefit of consuming carotenoids could help explain both the origin and the maintenance of the preference.

2. METHODS

(a) *Experimental design and carotenoid diets*

The guppies used in this study were the first- and second-generation descendants of fishes collected from four undisturbed streams in the Northern Range of Trinidad. Two of these streams are located in the upper Madamas drainage basin; the other two are located in the upper Quare drainage basin. The experimental design was completely balanced with respect to stream of origin and diet group, although the level of replication was inadequate for making stream comparisons (and this was not one of the goals of the study). In total, we used 48 mature males and 48 mature females fed one of two diets that were identical except for the concentration of carotenoids (low diet (in $\mu\text{g g}^{-1}$): 0.28 ± 0.56 lutein, 0.51 ± 0.73 β -carotene; and high diet (in $\mu\text{g g}^{-1}$): 969.4 ± 110.4 lutein, 93.8 ± 33.8 zeaxanthin, 1017.8 ± 370.0 β -carotene). Both diets were nutritionally complete, with vitamin A supplied in palmitate form. A specialized feeding device was used to deliver a precise amount of powdered food to each tank (approximately as much food as the fishes were willing to eat, adjusted for age and the number of fishes in the tank), twice a day on weekdays and once a day at weekends. All 96 fishes used in this experiment were maintained on the experimental diets for at least 10 weeks prior to the start of the experiment. Half of the females and all of the males had been maintained on these diets since birth. Females raised on the experimental diets from birth (group 2) were housed under slightly different conditions from the males and the rest of the females (group 1), as described below, but otherwise were treated identically.

The experiment involved transplanting individual scales between pairs of unrelated fishes to create reciprocal allografts (Hildemann 1957; Angus & Schultz 1979; see details in § 2b).

Fifteen days following the initial grafting, identical second-set transplants were exchanged between the same donors and recipients as the first transplants. Accelerated rejection of second-set grafts from the same donor is one of the hallmarks of adaptive (acquired) immunity (Cooper 1964).

The members of each reciprocal-transplant pair were from different genetically isolated streams in the same drainage basin, to eliminate the remote possibility that close relatives were accidentally paired. Prior to the experiment, the fishes were housed in 8 l plastic aquaria at densities of one female and one to four males. During the experiment, the fishes were housed in the same type of aquaria at a standardized density of one female and two males (group 1) or one female and one male (group 2). Within each aquarium, males were distinguished from each other based on colour-pattern differences (which we sketched at the start of the experiment). This ensured that the first and second allografts were performed between the same two fishes. All aquaria used in this experiment were placed on a single rack in a temperature-controlled (24.5 ± 1 °C) windowless room maintained on a 12 L : 12 D photoperiod, and interspersed with respect to carotenoid diet and stream of origin.

The first graft to one of the females from group 2 was dislodged soon after grafting, so the data for this graft were excluded from the analysis. One male died of unknown causes 4 days after the first allograft, but the remaining fishes survived and were added to our breeding colony at the end of the experiment.

(b) *Exchange and monitoring of allografts*

Scale (skin) allografts were performed according to the methods developed by Cooper (1964) (also see Hildemann 1957) using the mummichog, *Fundulus heteroclitus*. Guppies in a reciprocal pair were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester) and placed side-by-side on wet gauze under a microscope. Individual scales from a specific location (determined by counting back from the brain, which is visible through the skin) on the head of the donor were gently removed using watchmaker forceps and placed in the empty scale pocket of the recipient. Immediately after grafting, the fishes were allowed to recuperate quietly in a Petri dish with well-soaked gauze. After 5 min, when the grafts were settled enough for swimming, the fishes were returned to their respective aquaria.

Beginning on day one, and every day thereafter for 10 days, transplanted scales were observed under a microscope for evidence of healing, inflammation and other gross signs of incompatibility, using criteria modified from Cooper (1964), by an observer 'blind' to the diet group of the fishes being examined. See § 3a for further details on the allograft scoring method.

(c) *Data analysis*

To test for effects of carotenoid diet on the strength of the immune response, repeated measures ANOVAs (Tabachnick & Fidell 2001), with immune response as the dependent variable, time (days since transplant) as the repeated within-subjects factor and carotenoid diet (low versus high) and sex as the between-subjects factors, were performed separately by graft set (first versus second) using JMP 3.2.2 (SAS Institute Inc., Cary, NC, USA). We also performed a separate repeated-measures ANOVA to check for differences between the two female groups.

3. RESULTS

(a) *Morphological changes observed in allografted scales*

The morphological changes that we observed in transplanted scales consisted of swelling, cloudiness and disruption and disappearance of melanocytes. Previous studies of scale allografts in fishes have used the time to complete loss of melanocytes as a measure of immune-system strength (e.g. Angus & Schultz 1979; Dawley *et al.* 2000; Cardwell *et al.* 2001). In our study, however, melanocytes sometimes disappeared and later reappeared in the transplanted scales or, in other cases, were never observed to disappear completely. Our interpretation is that melanocytes in the allografted scales were destroyed and rapidly replaced by melanocyte-containing host tissue, obscuring the time of complete allograft rejection. In lieu of a consistently reliable method for determining the time to rejection, we constructed a composite 'rejection response' variable incorporating all of the observed morphological changes: level 0, slight swelling only; level 1, swelling or melanocytes disrupted; level 2, swelling and melanocytes disrupted; level 3, swelling, melanocytes disrupted and slight cloudiness; level 4, swelling, melanocytes disrupted or partially absent and cloudiness; and level 5, swelling, melanocytes absent and strong cloudiness. This response scale was devised prior to the start of the experiment, based on our preliminary observations. In a pilot study, control autograft scales showed no evidence of rejection, except for initial transient inflammatory responses (i.e. level 0). Thus, self tissue is always accepted without rejection (Cooper 1977).

(b) *Effect of carotenoid diets on the rejection response*

Although the two female groups (on the carotenoid diets since birth or for at least 10 weeks) differed in their levels of response (first graft: $F_{1,43} = 16.44$, $p = 0.0002$; second graft: $F_{1,43} = 12.01$, $p = 0.001$), there was no significant carotenoid-diet \times group interaction (first graft: $F_{1,43} = 0.99$, $p = 0.0001$; second graft: $F_{1,43} = 2.81$, $p = 0.10$), which indicates that the groups did not differ in the effect of carotenoids on the allograft rejection response. We therefore combined the results from the two female groups in subsequent analyses.

There were significant changes in the rejection response over time for all groups (first graft: $F_{9,82} = 29.91$, $p < 0.0001$; second graft: $F_{9,81} = 18.31$, $p < 0.0001$), with a peak response on days 3–5 (figure 1). However, the diet \times time interaction term was non-significant ($p > 0.10$). There was also a significant sex \times time interaction for both grafts (first graft: $F_{9,82} = 2.08$, $p = 0.040$; second graft: $F_{9,81} = 2.44$, $p = 0.017$). The level of carotenoids in the diet had no significant effect on the rejection response to the first allograft ($F_{1,90} = 0.02$, $p = 0.90$) but did have an effect on the rejection response to the second allograft ($F_{1,89} = 5.22$, $p = 0.025$). There was no significant sex difference in the rejection response (first graft: $F_{1,90} = 0.21$, $p = 0.65$; second graft: $F_{1,89} = 3.20$, $p = 0.077$). However, there was a significant sex \times diet interaction term for the second graft (first graft: $F_{1,90} = 0.23$, $p = 0.63$; second graft: $F_{1,89} = 5.10$, $p = 0.026$), suggesting that the diet effect was restricted to males (figure 1). To verify this conclusion, we ran separate

analyses by sex. The diet effect on the second graft was significant for males (first graft: $F_{1,45} = 0.07$, $p = 0.79$; second graft: $F_{1,44} = 7.43$, $p = 0.009$) but not for females (first graft: $F_{1,43} = 0.30$, $p = 0.59$; second graft: $F_{1,43} = 0.0001$, $p = 0.99$).

4. DISCUSSION

We found that male guppies raised on a high-carotenoid diet mounted a significantly stronger rejection response to second-set allografts than did males raised on a low-carotenoid diet. Apparently, carotenoids boost some (unidentified) component of the adaptive (acquired) immune system. This could explain why carotenoids appear to be mobilized from the skin of male guppies infected with *Gyrodactylus*, an ectoparasitic helminth that guppies encounter in the wild (Houde & Torio 1992). This is one of the first experimental demonstrations of the effects of dietary carotenoids on the immune system of a species with carotenoid coloration (also see Blount *et al.* 2003), and the first such study, to our knowledge, on a species known to be carotenoid limited in the wild (Grether *et al.* 1999).

Interestingly, the high-carotenoid diet did not enhance the ability of female guppies to reject allografts. One plausible explanation is that the low-carotenoid diet provides adequate amounts of carotenoids to support immune function in the absence of competition between the immune system and sexual coloration. Males may be faced with a carotenoid-allocation trade-off. The optimal allocation of carotenoids between sexual coloration and non-sexual tissues may leave males in low-carotenoid environments immunocompromised (Lozano 1994). This is a specific version of the general immunocompetence handicap hypothesis (Folstad & Karter 1992). Whether female preferences for carotenoid coloration have evolved to exploit the trade-off faced by males is a separate issue (discussed below).

Comparisons of guppies from high- and low-carotenoid-availability streams suggest that males are more carotenoid-limited than are females (Grether *et al.* 1999). The concentration of carotenoids in the skin of female guppies, which presumably serves non-sexual functions (e.g. protection from ultraviolet light, crypsis), is not significantly correlated with carotenoid availability in the wild, and the same is true for the skin of males outside the orange spots. Thus, guppies appear to be able to meet the utilitarian demands of carotenoid pigmentation even in low-carotenoid-availability streams. By contrast, the concentration of carotenoids in the orange spots of males is strongly influenced by carotenoid availability and can exceed that in the skin outside the orange spots by an order of magnitude or more (Grether *et al.* 1999). Although males consume less carotenoids than females do (even after adjusting for sex differences in body mass), they deposit a greater fraction of the ingested carotenoids into their skin (Hudon *et al.* 2003). Without a complete carotenoid budget (assimilated, deposited in skin, deposited in eggs, etc.) it would be premature to draw firm conclusions, but it seems possible that females face less of a carotenoid-allocation trade-off than males do.

The hypothesized trade-off between carotenoid use by the immune system and carotenoid deposition in the

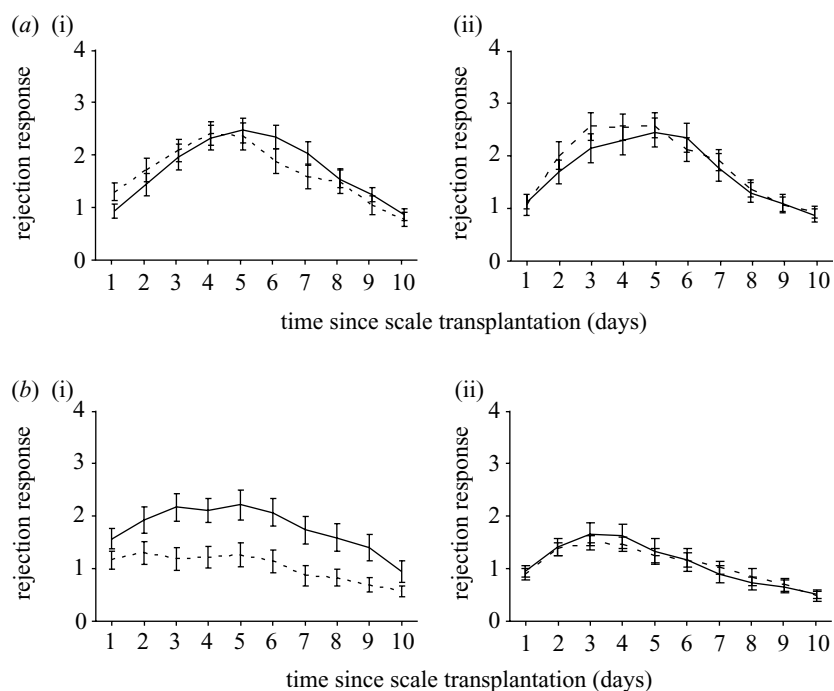


Figure 1. Response to allografted scales by male and female guppies (*Poecilia reticulata*) fed low-carotenoid (dashed lines) and high-carotenoid (solid lines) diets. (a) The mean response to the first-set allografts: (i) males and (ii) females; and (b) the mean response to the second-set allografts: (i) males and (ii) females. Bars show ± 1 s.e.

integument (Lozano 1994; Olson & Owens 1998; Møller *et al.* 2000) has been criticized on the grounds that the quantities of carotenoids required by the immune system may be trivial in comparison to the amounts required for sexual coloration (Hill 1999). Indeed, most of the experimental evidence for immune-system-enhancing effects of carotenoids has been obtained for mammals, which lack conspicuous carotenoid coloration and tend to have relatively low levels of circulating carotenoids (see table 1 in Hill 1999; but see Lozano 2001). The evidence for immune-system-enhancing effects of carotenoids in birds and fishes with carotenoid coloration (reviewed in Møller *et al.* 2000) is largely correlational and open to multiple interpretations (but see Blount *et al.* 2003; Faivre *et al.* 2003). One shortcoming of our study was that only two levels of dietary carotenoids were used. It is likely that the low-carotenoid diet was lower in carotenoids than the diet that guppies encounter in nature. Thus, we have not fully addressed Hill's (1999) criticism of Lozano's (1994) immunological trade-off hypothesis. Future studies should manipulate carotenoid intake over a wider range.

Moreover, we have probed only one component of the immune response of guppies. The vertebrate immune system is bilateral, consisting of innate immunity (retained from invertebrate ancestors) and adaptive or acquired immunity. Innate immunity operates through neutrophils and the various immune molecules that they synthesize and secrete in response to foreign material such as parasites and pathogens (Jones 2001). Adaptive immunity is associated with macrophages, T cells and B cells, which defend against foreign material including bacteria, viruses, parasites and experimentally introduced antigens. Our study was restricted to only a small part of the immune response, namely cellular responses to allografts. Future studies of the effects of carotenoid intake on immunity should examine variables such as antioxidant activity,

lysozyme activity, phagocytosis and response to naturally occurring parasites (Houde & Torio 1992; Lindenstrøm & Buchmann 2000; Köllner *et al.* 2002), with the goal of understanding the importance of immune-system effects of carotenoids in nature.

Nevertheless, the results of this study offer preliminary support for the hypothesis that the use of carotenoids in courtship displays is related to the immunological benefits of these pigments.

Females could potentially benefit in at least three ways from being attracted to carotenoid-based colours: first, through the effects of carotenoids on their own immune systems; second, by avoiding males with sexually transmitted diseases who have mobilized their skin carotenoids to combat infections (Houde & Torio 1992; Lozano 1994); and third, by choosing healthy mates and passing on good genes for disease resistance to their offspring (Hamilton & Zuk 1982; Folstad & Karter 1992; Lozano 1994). The first of these benefits could account for the evolutionary origin of carotenoid-coloration preferences, and the other two benefits could explain the maintenance and further elaboration of the preference. For guppies, however, the available evidence does not support either of these hypotheses. Neither the degree of attraction to orange objects nor the strength of the female preference for carotenoid coloration correlates with carotenoid availability in the wild (Grether 2000; Rodd *et al.* 2002). The only known geographical predictor of the strength of the mate preference for carotenoid coloration is the degree of attraction to orange objects, and vice versa ($r^2 = 0.94$; Rodd *et al.* 2002). We therefore have no evidence, as of yet, that the mate preference for carotenoid coloration evolved to exploit the relationship between carotenoid coloration and male health. This does not, however, undermine the hypothesis that males are faced with a trade-off between attracting mates and staying healthy.

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