# The carotenoid beta-carotene enhances facial colour, attractiveness and perceived health, but not actual health, in humans.

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Abstract

Carotenoid-based colouration plays an important role in mate choice in many animal species. It is argued to be an honest signal of health because carotenoids function as antioxidants and only healthy individuals can afford to use available carotenoids for signalling. Here, we tested the effect of dietary supplementation of the carotenoid beta-carotene on facial appearance and health in human males. Beta-carotene supplementation altered skin colour to increase facial attractiveness and perceived health. However, we found no effect of beta-carotene on measures of actual health, including oxidative stress, innate immune function, and semen quality. We conclude that although carotenoid-based skin colour may be sexually selected in human males, it may not be an honest signal of health.

*Key words*: carotenoid trade-off hypothesis, humans, mate choice, skin colour

## Introduction

In many species, some individuals are more attractive as mating partners compared to others (Darwin 1871; Andersson 1994). A number of mechanisms have been proposed to explain the evolution of mate choice (Andersson 1994). One is that attractive traits provide information on the biological quality of the signaller, for example on signaller health (Andersson 1994). Mating with healthy individuals can enhance reproductive fitness in a number of ways: a healthy mate may provide direct benefits in the form of better parenting, be less likely to be infected with pathogens that threaten survival, or have greater fertility (Møller & Jennions 2001). They may also provide indirect benefits in the form of genes that promote good health in offspring (Zahavi 1975; Hamilton & Zuk 1982). Another possibility is the Fisherian sexy son mechanism, where individuals with attractive mates produce offspring that enjoy greater reproductive success because they inherit their father's attractiveness (Fisher 1930; Prokop *et al.* 2012; Mitchem *et al.* 2013).

One attractive trait that plays an important role in sexual selection is carotenoid-based colouration (Svensson & Wong 2011). Carotenoids are red and yellow pigments present in the fruits and vegetables that animals consume (Alaluf *et al.* 2002). In many species these pigments are used in the expression of brightly coloured ornaments, from the yellow feathers of greenfinches (Hõrak *et al.* 2007), to the red-orange frills of Australian frillneck lizards (Hamilton *et al.* 2013) and the bright orange spots of guppies (Grether *et al.* 1999). Early evidence of an effect of carotenoids on mate choice in non-human animals came from work on guppies, *Poecilia reticulata*, for which male carotenoid colouration was found to predict female responses to courting males and their probability of copulation (Endler 1983). Since then, similar results have been found in various species of birds, fishes and

reptiles (Kodric-Brown 1983; Olson & Owens 1998; Møller *et al.* 2000; Simons & Verhulst 2011; Blount 2004; Kwiatkowski & Sullivan 2002; Fitze *et al.* 2009).

For sexual signals to honestly signal health, there has to be a cost so that unhealthy individuals are unable to "cheat" by expressing those ornaments (Zahavi 1975). According to the carotenoid trade-off hypothesis, this cost arises from trade-offs between using carotenoids for colouration and using them as antioxidants to quench reactive oxygen species (ROS) (Lozano 1994; von Schantz *et al.* 1999). ROS are by-products formed during the respiratory metabolism of oxygen (Halliwell & Gutteridge). At low levels, ROS can facilitate biological processes such as immune defence (Nappi & Ottaviani 2000). However, excess production of ROS causes oxidative damage to cells and DNA. Important health functions such as immune function and semen quality are highly susceptible to oxidative damage because immune and sperm cells contain high amounts of polyunsaturated fats (Knight 2000; Dowling & Simmons 2009). Therefore, antioxidants are required to protect the organism from the damaging properties of ROS. Carotenoids have been shown to be capable of scavenging ROS *in vitro* (Young & Lowe 2001). Therefore, it has been suggested that besides enhancing colouration, carotenoids may also affect health *in vivo* by reducing oxidative stress, supporting immune responses, and maintaining semen quality. According to the carotenoid trade-off hypothesis (Lozano 1994; von Schantz *et al.* 1999), because carotenoids can only be obtained through fruits and vegetables in the diet, they represent a limited resource that must be allocated to signalling versus antioxidant function depending on current need. Consequently, this trade-off leads to a positive relationship between carotenoid colouration and heath: healthy individuals are able to use available carotenoids for colouration while unhealthy individuals have to reserve available carotenoids for quenching ROS (Lozano 1994; von Schantz *et al.* 1999).

The hypothesis that carotenoids signal health has received empirical support. Correlational studies demonstrate that carotenoids and carotenoid colouration can be positively correlated with measures of health. For example, a meta-analysis of studies across 88 species of birds reported that circulating carotenoids are positively correlated with immune function and oxidative stress, and carotenoid colouration is positively related to immune function (Simons *et al.* 2012). In blue tits, *Parus major*, carotenoid colouration is positively related to the sperm's ability to resist the harmful effects of an oxidative challenge (Helfenstein *et al.* 2010), and in mallards, *Anas platyrhynchos*, carotenoid-based bill colour is an indicator of both immune function and sperm performance (Peters *et al.* 2004). In humans, consumption of carotenoid rich foods has been associated with increased semen quality and improved male fertility (Zareba *et al.* 2013). Furthermore, immune and oxidative stress challenges can result in a reduction in carotenoid pigments of colouration in birds and lizards (e.g. Aguilera & Amat 2007; Fitze *et al.* 2007; López *et al.* 2009; Alonso-Álvarez & Galván 2011). These studies indicate that carotenoid colouration may be dependent on an individual's health and condition.

Despite correlational findings linking carotenoids to health and *in vitro* evidence supporting the key assumption of the carotenoid trade-off hypothesis that carotenoids affect health, a role for carotenoids in health *in vivo* remains contentious. Several researchers have suggested that carotenoids are at best minor antioxidants (Halliwell 1999; Hartley & Kennedy 2004; Costantini & Møller 2008; Pérez-Rodríguez 2009). Costantini and Møller (2008) conducted a meta-analytic review and concluded that there is little evidence that carotenoids function as antioxidants in birds. As for the effects of carotenoids on immune function, although some animal studies have found positive effects (e.g. Aguilera & Amat 2007; Blount *et al.* 2003; Grether *et al.* 2004; McGraw & Ardia 2003), others have found null or even negative effects (null

findings: Fitze *et al.* 2007; Navara & Hill 2003; Lin *et al.* 2010; McGraw & Klasing 2006); negative effects: Sild *et al.* 2011). Less research has been done on the effect of carotenoids on male reproductive health, and these findings are equally unclear. In three-spined sticklebacks, *Gasterosteus aculeatus*, for example, males that were fed higher amounts of carotenoids fertilized more eggs when provided with a clutch of unfertilized eggs (Pike *et al.* 2010). However, in a competitive fertilization study of crickets, *Teleogryllus oceanicus*, the carotenoid beta-carotene did not affect sperm competitiveness unless it was consumed together with vitamin E (Almbro *et al.* 2011).

Although the role carotenoids play in mate choice is well-established in taxonomic groups such as birds and fishes, the evidence in mammals has been lacking, partly because colour-based sexual signalling is relatively rare in mammals as many species lack trichromatic colour vision (Changizi *et al.* 2006). Emerging evidence using the CIELab colour space, which models human trichromatic colour vision based on three axes, namely lightness, redness, and yellowness, suggests that carotenoids may affect mate choice in humans by influencing their facial skin colour, particularly yellowness. First, skin yellowness may be correlated with carotenoid intake. Individual variation in beta-carotene intake based on self-reported fruit and vegetable consumption was found to be positively related to skin yellowness (Stephen *et al.* 2011). In addition, changes in self-reported fruit and vegetable intake over six weeks were found to be positively related to changes in skin yellowness, with the spectral reflectance changes directly related to the spectral absorption of carotenoids (Whitehead *et al.* 2012). Moreover, a recent intervention study found that consumption of carotenoid-rich fruit and vegetable smoothies over six weeks increased skin yellowness and redness (Tan *et al.* 2015). Second, carotenoid-based skin colour is associated with attractiveness in humans. Individuals preferred faces

that were transformed to be high in carotenoid colour over low-carotenoid-colour versions of the same faces (Lefevre *et al.* 2013; Lefevre & Perrett 2014). Skin yellowness is also related to attractiveness of own-race faces for both Caucasian and African participants (Stephen *et al.* 2012). Facial yellowness is also related to how healthy faces appear, which is closely linked to attractiveness (Rhodes *et al.* 2007). Both Caucasian and African participants increased yellowness in own-race face images when asked to adjust the colour to make the faces look healthier (Stephen *et al.* 2011; Stephen *et al.* 2009). Moreover, these preferences appear to be specific to skin colour, suggesting a special salience for skin colouration, rather than reflecting a general sensory bias for yellow/red colour (Lefevre *et al.* 2013; Tan & Stephen 2013).

There are, however, several limitations in the human carotenoid signalling literature. The evidence relating carotenoids to human facial appearance is almost entirely correlational, which prevents conclusions of causation. Two studies reported that participants given daily beta-carotene supplements for eight weeks showed an increase in skin yellowness and redness (Stephen *et al.* 2011; Coetzee & Perrett 2014). However, due to the lack of a control group in both studies, we cannot exclude the possibility that the observed colour changes were due to factors other than beta-carotene supplementation. Furthermore, both studies had a very small sample size of 10, which makes it difficult to draw firm conclusions. Tan *et al.* (2015) found a significant effect of consuming carotenoid-rich fruit and vegetable smoothies on skin colour, compared to a placebo condition of drinking filtered water. However, we can’t be certain that the effect was due to carotenoids and not to other nutrients in the smoothies. No studies have tested experimentally the effects of carotenoids on attractiveness and perceived health. Most importantly, no experimental study has tested whether carotenoids improve actual health as predicted by the carotenoid trade-off hypothesis.

Here we use a randomized, double-blind, placebo-controlled supplementation study to investigate the effects of beta-carotene on facial appearance and health in humans. We focus our study on female preferences for male appearance. First, we establish the effect of carotenoids on face colour. Based on previous findings (Stephen *et al.* 2011; Tan *et al.* 2015), we hypothesize that beta-carotene will enhance facial yellowness and redness. Second, we examine the effect of beta-carotene on attractiveness. We hypothesize that beta-carotene supplementation will enhance facial attractiveness. Together, the two hypothesized results would support the idea that carotenoids play a role in human sexual selection by altering skin colour. Third, we examine the effect of beta-carotene on healthy appearance. Based on previous findings that skin yellowness is positively correlated with perceived health (Stephen *et al.* 2011;Stephen *et al.* 2009), we hypothesize that beta-carotene supplementation will enhance perceived health. Finally, we examine whether beta-carotene enhances actual health. We investigate a range of physiological health measures that are theoretically linked to carotenoids, including oxidative stress, innate immune function, and semen quality. A positive effect of beta-carotene on any of these health measures would support the idea that carotenoid colouration is an honest signal of health.

## Methods

### Ethics Statement

The study was approved by the Human Ethics Committee at the University of Western Australia (Ethics approval ref. no. RA/4/1/5909). All participants provided written consent prior to their participation in the project.

### Participants

Forty-three Caucasian men with a mean age of 21 years 11 months ($M$ = 21.93, $SD$ = 4.23) were recruited for the supplementation study from The University of X community. Each of them received either course credit or transport remuneration. All of them identified themselves as heterosexual and reported that they did not suffer from any immunological, endocrine, or metabolic disorders. Two previous studies that investigated the effect of beta-carotene supplementation on skin colour have found significant changes in skin colour after eight weeks of beta-carotene supplementation with 10 participants and neither study had a placebo condition (Stephen *et al.* 2011; Coetzee & Perrett 2014). The sample size in our treatment condition ($N_{\text{beta-carotene}}$ = 23) was more than twice that in previous studies, and was close to the 2.5 times that Simonsohn (2015) recommended for replications. We also had a placebo group consisting of an additional 20 male participants.

## Procedure

### Pre-supplementation.

Participants first attended a 1.5-hour laboratory session, which was held between 12pm and 6pm to reduce any potential changes in the physiological variables to be measured that might arise due to circadian rhythm. They were asked to refrain from consuming any food or flavoured drinks one hour before the session, not wear any make-up or tanning agents, and be clean-shaven. Urine (10 ml) was first collected in a sterile bottle for oxidative stress measures. Saliva (5 ml) was then collected for immune function measures. Participants collected the saliva in a sterile bottle using the passive drool method after rinsing their mouth with water and waiting approximately 15 minutes. The urine and saliva samples were stored immediately in a 4°C fridge and transferred to a −80°C freezer within 4 hours of collection.

Front view photographs of the participants' faces, displaying a neutral expression with mouth closed, were taken under standardized symmetric lighting

conditions using a Nikon D7000. The photographs were taken in Nikon's proprietary NEF raw image format. Participants were seated at a fixed distance (130 cm) from the camera against a grey fabric background. A cape in the same grey fabric was draped over the participants to control for the colour of their clothing. Spectacles were removed and fringes covering the forehead were pulled back using a hairband. An X-rite Classic ColourChecker chart (Grand Rapids, MI, U.S.A) was placed next to the participants' faces for colour calibration purposes.

At the end of the laboratory session, the participants were given written instructions for semen collection, a sterile sample vial to collect the ejaculate, an ejaculate questionnaire, a piece of aluminium foil, and four pictures, each containing the front view image of a naked woman from Thornhill & Grammer (1999). They were asked to abstain from any ejaculation for two to six days before collecting the sample at home via masturbation while viewing the four images. The images provided visual stimulation, which is necessary for the production of a normal ejaculate (Wylie & Pacey 2011). Participants were asked to deliver the sample to the laboratory within one hour of collection. During delivery, they were asked to wrap the sample vial in aluminium foil and place it under their arm or between their legs to maintain its temperature. Participants also returned the completed ejaculate questionnaire, which noted the time at which the sample was collected, whether the entire ejaculate was collected and if not, the percentage and portion (initial, middle, or end) lost, and the time since their previous ejaculation.

After the participants delivered the semen sample, they were given a 12-week supplementation of either beta-carotene from Natural Factors (30000 IU per day) or lactose capsules (400 mg of 100% pure lactose per capsule). To ensure double blinding, both capsule types were stored in identical opaque bottles with tamper

evident seals that were broken only by the participants. The bottles were randomly assigned and coded by a research assistant who was not involved in the study.

**Post-supplementation.**

The participants returned after 12 weeks for the post-supplementation follow-up, where the photography and sample collection were repeated. The 12-week supplementation period was chosen because human spermatogenesis takes around 74 days and epididymal transit takes an additional 8 days, making a total of 82 days (~11.7 weeks) (Amann 2008).

**Face Colour Measurement**

The NEF files were converted to raw DNG files using Adobe's DNG Converter and then converted to lossless PNG files in Photoshop CS3. To control for random fluctuations in lighting conditions and camera settings, the photos were colour-calibrated by standardizing the colour of the ColourChecker patches based on known CIELab values using the colour calibration plugin in the program Psychomorph (Tiddeman & Perrett 2002).

The PNG files (4928 x 3264 pixels, 72 pixels/inch) were used to measure face colour using the software ImageJ (http://imagej.nih.gov/ij/). The measurements were taken from ten 60 x 60 pixels squares. Two squares were placed above each eyebrow on the forehead. Three squares were placed on each side of the cheek. Care was taken to avoid skin regions with blemishes, specular highlights, or shadows. Average RGB values of each square were extracted using the Color Histogram plugin in ImageJ and then converted to CIELab values using the formulas from the website EasyRGB (http://www.easyrgb.com/index.php?X=MATH). The CIELab values were moderately to highly repeatable across the 10 squares (lightness L*: $R = 0.59$, 95% CI [0.52, 0.66]; redness a*: $R = 0.62$, 95% CI [0.55, 0.69]; yellowness b*: $R = 0.73$, 95%

CI [0.68, 0.78]). The CIELab values were averaged across the ten squares to derive average lightness, redness, and yellowness values for each face.

**Facial Attractiveness And Perceived Health Pre- *Vs* Post-Supplementation**

Sixty-six self-reported heterosexual Caucasian female raters with a mean age of 33 years 2 months ($M$ = 33.13, $SD$ = 7.65) were recruited online via Amazon Mechanical Turk to assess the attractiveness and perceived health of the pre- and post-supplementation faces. Thirty-three raters with a mean age of 33 years 1 month ($M$ = 33.09, $SD$ = 7.70) assessed attractiveness and 33 raters with a mean age of 33 years 2 months ($M$ = 33.18, $SD$ = 7.72) assessed perceived health. We compared the attractiveness and perceived health of the pre- and post-supplementation colour-calibrated faces using a 2-alternative forced-choice procedure. In each trial, raters saw the pre- and post-supplementation faces of each male participant presented side by side on a computer screen. Raters had to select the more attractive or healthy looking face. Each face pair remained onscreen until the rater responded. Each task consisted of two blocks. All 43 male face pairs were presented twice, once in each block, making a total of 86 trials for each task. In block one, the post-supplementation face was shown on the right for half the face pairs. The face pairs were presented again in block two in the opposite left-right orientation. Block order was counterbalanced across participants. All faces were rotated and aligned so that the eyes were lying on a horizontal plane at the same height. All images were cropped to 372 x 491 pixels and a black oval mask was applied to cover most of the hair, ears, and neck. There was high inter-rater reliability with Cronbach's alpha of 0.91 and 0.92 for the attractiveness and perceived health tasks, respectively. An attractiveness score for each face pair was calculated as the percentage of times the post-supplementation face was chosen as the more attractive face. A perceived health score for each face pair

was calculated as the percentage of times the post-supplementation face was chosen as the more healthy looking face.

One concern regarding using online samples for the study of skin colour preferences is that the raters' computers are not colour-calibrated, which might introduce noise to the colour representation onscreen. However, several previous studies have investigated skin colour preferences using online samples (Lefevre *et al.* 2013; Lefevre & Perrett 2014; Carrito *et al.* 2016) and results from online studies agree with those from laboratory studies using colour-calibrated monitors (Lefevre & Perrett 2014). Both samples showed a preference for high carotenoid skin colour, with no difference in the preference between the two. This finding suggests that any additional noise due to un-calibrated monitors is relatively small compared to the colour variation among the faces.

**Urinary Oxidative Stress Assays**

Markers of DNA oxidation (8-OHdG) and lipid peroxidation (isoprostane) were analysed in duplicates using competitive enzyme-linked immunoassay (ELISA) kits from Northwest Life Science Specialties (Vancouver, VA, U.S.A.). A significant proportion of urinary isoprostane is conjugated to glucoronic acid, which is not assayable (Yan *et al.* 2010). To obtain a more accurate measure of overall isoprostane level, 100 µl of each sample was incubated with 5 µl of beta-glucoronidase for two hours at 37°C to cleave and free the isoprostanes from their conjugated forms before running the isoprostane assays. The 8-OHdG and isoprostane results were standardized against urinary creatinine levels (presented as ng/mg creatinine) to control for variation in urine concentration. Creatinine was determined in duplicates using colorimetric assay kits from Northwest Life Science Specialties (Vancouver, VA, U.S.A.). The 8-OHdG, isoprostane, and creatinine assays were highly repeatable

(8-OHdG: $R$ = 0.98, 95% CI [0.97, 0.99]; isoprostane: $R$ = 0.92, 95% CI [0.89, 0.95]; creatinine: $R$ = 0.99, 95% CI [0.99, 0.99]).

**Salivary Innate Immune Function Assays**

**Bacteria killing capacity.**

Salivary bacteria killing capacity against *Escherichia coli* (ATCC no. 8739) was analysed in triplicates using a published protocol (Prall & Muehlenbein 2015) similar to that used widely in animal studies with blood samples (Millet *et al.* 2007). Salivary supernatant was incubated with *E.coli* for 30 mins to facilitate bacteria killing, and then incubated overnight on trypticase soy agar (TSA) plates to quantify the amount of bacteria remaining (see supplementary material for details). Images of the plates were taken together with a ruler as a size reference. We used the program ImageJ to measure the following: total number of colonies in each plate, average area of each colony, and total area of the colonies combined. All three measures were highly repeatable (colony number: $R$ = 0.89, 95% CI [0.86, 0.92]; average colony area: $R$ = 0.89, 95% CI [0.85, 0.92]; total colony area: $R$ = 0.92, 95% CI [0.90, 0.95]). Bacteria killing capacity was calculated as the percentage difference in colony number relative to positive controls. Bacteria growth suppression capacity was calculated as the percentage difference in average colony area relative to positive controls. Overall salivary immunity was calculated as the percentage difference in total colony area relative to positive controls.

**Lysozyme activity.**

A lysoplate assay was used to determine salivary lysozyme activity. *Micrococcus lysodeikticus* (ATCC no. 4698) was reconstituted with PBS. Ten microlitres of whole saliva from each sample were added to 80µl of *M.lysodeikticus* in duplicates in a 96-well plate. Positive controls containing 10 µl of PBS and 80 µl of *M.lysodeikticus* were also added to the plate in duplicates. The plate was incubated at

33°C for 10 minutes and the absorbance was measured using an M5 SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance was highly repeatable ($R$ = 0.96, 95% CI [0.95, 0.98]). Salivary lysozyme activity was calculated as the difference in absorbance between the sample wells and the positive controls.

**Semen Analysis**

The semen samples were analysed in six replicates immediately upon delivery using the Hamilton-Thorne CEROS Computer Assisted Semen Analysis (CASA) system (Simmons *et al.* 2011). The system measures total sperm concentration, percentage motile sperm and seven motility related variables. Seven samples had to be diluted because they were too concentrated for the CASA to analyse (see supplementary material for details).

A portion of the post-supplementation semen sample was stored in accordance with (McEvoy *et al.* 2014) to analyse oxidative damage to sperm via the degree of DNA fragmentation (McEvoy *et al.* 2014) in duplicates using Halosperm G2 kits from Halotech DNA (Madrid, Spain). The semen DNA fragmentation level of each sample is measured by the percentage of fragmented sperm cells (see supplementary material for details). The percentage fragmented sperm was highly repeatable ($R$ = 0.94, 95% CI [0.90, 0.97]).

**Data Reduction And Quality Control**

Principal components analysis (PCA) was used to summarize the inter-related semen quality data and immune function data.

The immune function PCA returned two PCs (eigenvalues >1) that accounted for 79.8% of variation in immune function (Table S1). PC1 was weighted most strongly by bacterial killing and suppression capacity. PC2 was weighted most strongly by lysozyme activity and overall bacteria immunity.

The semen quality PCA returned three principal components (PCs) (eigenvalue > 1) that accounted for 87.2% of variation in semen quality (Table S2). PC1 was weighted most strongly by variables related to rapid progressive motility. PC2 was weighted most strongly by variables related to the linearity of the sperm movement. PC3 was weighted most strongly by high sperm concentration and percentage motile sperm with low levels of left-right head movement. PC3 was found to be influenced by variation in the collection procedure and abnormalities in the sample (WHO 2010) (see supplementary material for details). Therefore, we ran all analyses for PC3 on the residuals after accounting for these variables.

## Results

Difference scores were calculated for each of the three skin colour variables (yellowness, redness, and lightness), the two oxidative stress measures (8-OHdG and isoprostane), the two immune function PCs, and the three semen quality PCs, by subtracting the pre-supplementation scores from the post-supplementation scores. The descriptive statistics for the dependent variables, including all the difference scores, 2-alternative forced-choice attractiveness scores, 2-alternative forced-choice perceived health scores, and post-supplementation sperm DNA fragmentation levels are presented in Table 1. Positive values indicate an increase in a particular measure post-supplementation. Separate pre- and post-supplementation descriptive statistics (*N*s, *M*s, *SD*s, and 95% CIs) for all dependent variables are presented in Table S3. During data analysis, the treatment conditions were binary coded to prevent any experimenter biases and the code was broken only after the statistical analyses were finalized. One participant's data were excluded from all analyses because his follow up sessions were delayed for more than 6 weeks, leaving an *N* of 42.

One-way ANOVAs with Treatment (beta-carotene, placebo) as the between-participants factor were conducted for all the dependent variables, including each of the skin colour difference scores (yellowness, redness, lightness), the 2-alternative forced-choice attractiveness score, the 2-alternative forced-choice perceived health score, each of the oxidative stress difference scores, each of the immune function PC difference scores, each of the semen quality PCs and the post-supplementation sperm DNA fragmentation levels. All residuals were normally distributed.

**Facial Appearance**

As hypothesized, there was a significant effect of Treatment on changes in face yellowness ($F_{1,40}$ = 23.31, $p$ < 0.001, $d$ = 1.50) (Table 1; Figure 1). One sample t-tests showed a significant post-supplementation increase in skin yellowness for the beta-carotene group ($t_{22}$ = 7.54, $p$ < 0.001), but not the placebo group ($t_{18}$ = 1.27, $p$ = 0.22). These results indicate that the Treatment effect was due to an increase in yellowness in the beta-carotene group. Also, as hypothesized, there was a significant effect of Treatment on changes in face redness ($F_{1,40}$ = 6.22, $p$ = 0.02, $d$ = 0.77) (Table 1; Figure 1). Again, the post-supplementation increase was significant for the beta-carotene group ($t_{22}$ = 2.67, $p$ = 0.01), but not the placebo group ($t_{18}$ = 1.22, $p$ = 0.24), indicating that the Treatment effect was due to an increase in redness in the beta-carotene group. There was no significant effect of Treatment on changes in face lightness ($F_{1,40}$ = 0.25, $p$ = 0.62, $d$ = 0.15) (Table 1; Figure 1). These results show that, as predicted, beta-carotene supplementation significantly increased face yellowness and redness but not lightness. Examples of the colour variation between pre- and post-supplementation for the beta-carotene and placebo groups are shown in Figure 2.

There was a significant effect of Treatment on the proportion of post-supplementation faces chosen as more attractive looking ($F_{1,40}$ = 9.98, $p$ = 0.003, $d$ =

0.98) (Table 1; Figure 3). Post-supplementation faces were chosen as more attractive than the pre-supplementation faces significantly above 50% chance level in the beta-carotene treatment group ($t_{22}$ = 3.85, $p$ = 0.001), but not in the placebo treatment group ($t_{18}$ = 1.13, $p$ = 0.27). Thus beta-carotene supplementation significantly enhanced facial attractiveness.

There was a significant effect of Treatment on the proportion of post-supplementation faces chosen as healthier looking ($F_{1,40}$ = 5.72, $p$ = 0.02, $d$ = 0.74) (Table 1; Figure 3). Post-supplementation faces were chosen as healthier looking than the pre-supplementation faces significantly above 50% chance level in the beta-carotene treatment group ($t_{22}$ = 2.38, $p$ = 0.03) but not in the placebo treatment group ($t_{18}$ = 1.13, $p$ = 0.28). Thus beta-carotene supplementation significantly enhanced perceived health.

**Oxidative Stress**

One participant's 8-OHdG data was excluded because his pre-supplementation 8-OHdG level was too low to be measured. Treatment did not significantly affect changes in either 8-OHdG or isoprostane level (8-OHdG: $F_{1,39}$ = 0.56, $p$ = 0.46, $d$ = 0.32; isoprostane: $F_{1,40}$ = 0.04, $p$ = 0.85, $d$ = 0.10) (Table 1).

**Immune Function**

One participant's data was excluded for both PCs because his saliva sample did not contain sufficient supernatant for us to run the bacterial killing capacity assay. Treatment did not significantly affect changes in either of the immune function PCs (PC1: $F_{1,39}$ = 1.65, $p$ = 0.21, $d$ = 0.40; PC2: $F_{1,39}$ = 0.14, $p$ = 0.71, $d$ = 0.12) (Table 1).

**Semen Analysis**

Two participants' data were excluded from all the semen analyses because their sperm concentrations were below the lower reference limit for normal samples according to the WHO (2010). Three participants' PC3 data were excluded because of

missing ejaculate questionnaire items that we used to extract the residuals for PC3. One participant's sperm DNA fragmentation data was missing due to technical errors when running the analysis. Treatment did not significantly affect changes in any of the semen quality PCs, or the sperm DNA fragmentation levels (PC1: $F_{1,38} = 1.01$, $p = 0.32$, $d = 0.32$; PC2: $F_{1,38} = 0.54$, $p = 0.27$, $d = 0.24$; residualized PC3: $F_{1,35} = 2.28$, $p = 0.14$, $d = 0.50$; sperm DNA fragmentation levels: $F_{1,37} = 1.12$, $p = 0.30$, $d = 0.34$) (Table 1).

## Discussion

We provide experimental evidence that the carotenoid beta-carotene enhances skin yellowness and redness and increases facial attractiveness in human males. Contrary to the carotenoid trade-off hypothesis, we did not find any effect of beta-carotene on measures of oxidative stress, immune function, semen quality, or sperm DNA fragmentation. Thus, despite the effects of beta-carotene on facial appearance, we find no evidence that carotenoid-related skin color is an honest signal of health in human males.

Carotenoid-based colouration has been shown to influence mate choice in taxa such as birds, fishes, and lizards (Endler 1983; Kodric-Brown 1983; Olson & Owens 1998; Møller *et al.* 2000; Simons & Verhulst 2011; Blount 2004; Kwiatkowski & Sullivan 2002; Fitze *et al.* 2009), but evidence in mammals is lacking. Our results suggest that carotenoid-based colouration also serves mate choice functions in humans. First, using a randomized, double-blind, placebo-controlled experimental design, we provide strong experimental evidence that consuming beta-carotene enhances skin yellowness and redness. Second, we showed that there was a significant effect of beta-carotene supplementation on male facial attractiveness and perceived health. Recent correlational studies have linked beta-carotene intake with

increased skin yellowness (Stephen *et al.* 2011; Whitehead *et al.* 2012; Tan *et al.* 2015), facial attractiveness (Lefevre *et al.* 2013; Lefevre & Perrett 2014; Stephen *et al.* 2012) and perceived health (Stephen *et al.* 2011; Stephen *et al.* 2009). Our study provides the first evidence for a causal link between beta-carotene and these changes. To the extent that attractiveness affects mating success (Rhodes *et al.* 2007), our results suggest that carotenoid-based skin colour may be sexually selected in human males.

According to the carotenoid trade-off hypothesis, colouration signals health because individuals face a trade-off between the use of available carotenoids in colouration *vs* supporting health. The assumption that carotenoids affect health has been tested experimentally in numerous species of birds, fishes, lizards, and even insects, but the results have been equivocal (Aguilera & Amat 2007; Blount *et al.* 2003; Grether *et al.* 2004; McGraw & Ardia 2003; Fitze *et al.* 2007; Navara & Hill 2003; Lin *et al.* 2010; McGraw & Klasing 2006; Sild *et al.* 2011). For humans, we found that although beta-carotene made the participants look healthier, there was no evidence that it enhanced actual health. Beta-carotene supplementation did not affect innate immune function, oxidative stress, or semen quality, all measures that have been linked theoretically to the proposed antioxidant capacity of carotenoids. Moreover, for each aspect of health, we used multiple measures, which should be superior to using single measures (Adamo 2004; Halliwell & Whiteman 2004). Our results suggest that, rather than indicating actual health changes, the changes in perceived health due to beta-carotene supplementation may reflect an attractiveness halo effect (Eagly *et al.* 1991).

It is possible that beta-carotene supplementation might have an effect on health in a population that is under greater physiological health stress or greater dietary restrictions than our sample. From a life-history perspective, physiological trade-offs

are more apparent when individual or environmental conditions are limiting (Stearns 1977). Our participants were all relatively healthy individuals recruited from a university community. It is possible that we found an effect of beta-carotene on facial appearance but not health because the participants simply did not require additional carotenoids to support their health and devoted all the supplemented beta-carotene to appearance. It would be informative for future studies to examine a population that is under greater physiological or dietary stress.

Although we did not find a significant effect of beta-carotene on any of our health measures, carotenoids could still be linked to health via indirect mechanisms. For example, Hartley and Kennedy (2004) postulated that carotenoid colouration might actually signal the presence of other antioxidants that protect carotenoids from oxidative damage, which would otherwise cause carotenoids to lose their colour (i.e. become bleached) and thus reduce the intensity of carotenoid signals. This hypothesis is also known as the carotenoid protection hypothesis. In support of this hypothesis, experimental studies have found that dietary supplementation of non-pigmentary antioxidants increase carotenoid-based colouration in species such as zebra finches, *Taeniopygia guttata* (Bertrand *et al.* 2006), three-spined sticklebacks, *Gasterosteus aculeatus* (Pike *et al.* 2007), and yellow-legged gulls, *Larus michahellis* (Pérez *et al.* 2008). It would be interesting to examine the carotenoid protection hypothesis in humans by investigating the effect of consuming non-pigmentary antioxidants on skin yellowness and redness.

Another possibility is that carotenoids only affect health when they are paired with other nutrients. Almbro *et al.* (2011), for example, showed that beta-carotene increased sperm competitiveness of male crickets, *Teleogryllus oceanicus*, only when it was taken together with vitamin E. They argued that because vitamin E is converted to radical species when it is used as an antioxidant, beta-carotene might serve to

recycle the radicalised vitamin E, thus enhancing overall antioxidant status. Therefore, a potential future direction would be to examine whether beta-carotene affects health in humans when paired with vitamin E.

In most species, carotenoid-based colouration is sexually selected via female mate choice for male carotenoid ornamentation, and most species show sexual dimorphism in carotenoid colouration. A recent study reported that human skin colour is also sexually dimorphic (Carrito *et al.* 2016). However, mate selection in humans occurs in both directions and carotenoids influence appearance in both sexes (Stephen *et al.* 2011; Whitehead *et al.* 2012; Tan *et al.* 2015). Therefore, it would be interesting to see the extent to which our findings could be replicated in women.

It would also be interesting for future studies to examine whether beta-carotene influences health over a longer supplementation period. We chose the 12-week duration partly because spermatogenesis plus epididymal transit in humans takes a total of 82 days (~ 11.7 weeks) (Amann, 2008). Therefore, we should have been able to observe any effects of beta-carotene on semen quality after 12 weeks of supplementation. Previous studies examining the effects of antioxidant supplementation on semen quality and oxidative stress have used similar supplementation durations (Møller & Loft, 2002; Showell *et al.* 2011; Chen *et al.*, 2013; Kumalic & Pinter, 2014). However, it remains possible that we might observe significant effects of beta-carotene on health in a relatively healthy population like the one in the present study with a longer supplementation duration. Zareba *et. al.* (2008) found that semen quality was positively related to carotenoid intake estimated from a food frequency questionnaire that asked participants to report their food, beverage, and supplement consumption over the past year. Given this finding, it would be interesting to examine the long-term effect of beta-carotene supplementation on health by repeating the present study with a supplementation period of 1 year or more.

In summary, we report the first double-blind, placebo-controlled experimental study on the effect of carotenoids on human facial appearance and health. We found that beta-carotene alters skin colour by enhancing yellowness and redness to enhance facial attractiveness in human males. However, we found no evidence that carotenoids improve actual health. Together, our results suggest that carotenoid-based colouration may have been sexually selected in humans, but we have no evidence to suggest that it is an honest signal of health.

## Authors' contributions

YZF designed the study, conducted the experiment, analysed the data, interpreted the results and drafted the manuscript. GR and LWS were involved in designing the study, interpreting the results, and revising the manuscript.

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## Competing interests

We do not have any competing interests.

## Data accessibility

Analyses reported in this article can be reproduced using the data provided by Foo, Rhodes & Simmons (2016).

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768

769 **Figure captions**

770 Figure 1. Boxplots of pre-post changes in skin yellowness, redness, and lightness by
771 Treatment (Beta-carotene *vs* Placebo).

772 Figure 2. Examples of colour variation between pre- and post-supplementation by
773 Treatment condition. To preserve the anonymity of the participants, average
774 pre- and post-supplementation images were created from all the individual
775 identities in each condition ($N_{\text{beta-carotene}} = 23$, $N_{\text{placebo}} = 19$).

776 Figure 3. Boxplots of 2-alternative-forced-choice scores for attractiveness and
777 perceived health by Treatment (Beta-carotene *vs* Placebo).

778 Table 1. Means, SDs, and 95% confidence intervals for the dependent variables by
779 treatment condition. All variables are presented as pre-post difference scores
780 (indicated by Δ) with the exception of the 2-alternative forced-choice attractiveness
781 and perceived health scores, which were scored based on the percentage of times the
782 post-supplementation face of a participant was chosen as more attractive or healthy
783 looking respectively, and the sperm DNA fragmentation levels, which was measured
784 post-supplementation.

| | Beta-carotene group | | Placebo group | |
|---|---|---|---|---|
| | *M* (*SD*) [CI.lb, CI.ub] | *N* | *M* (*SD*) [CI.lb, CI.ub] | *N* |
| Face colour and appearance | | | | |
| Δ Yellowness b* | 2.70 (1.72) [1.96, 3.44] | 23 | 0.38 (1.31) [−0.25, 1.01] | 19 |
| Δ Redness a* | 0.55 (0.99) [0.12, 0.97] | 23 | −0.43 (1.54) [−1.17, 0.31] | 19 |
| Δ Lightness L* | −0.72 (1.93) [−1.55, 0.12] | 23 | −0.39 (2.27) [−1.49, 0.70] | 19 |
| Attractiveness % | 60.6 (13.2) [54.9, 66.3] | 23 | 45.3 (18.2) [36.5, 54.0] | 19 |
| Perceived health % | 57.8 (15.7) [51.0, 64.6] | 23 | 45.4 (17.9) [36.7, 54.0] | 19 |
| Oxidative stress | | | | |
| Δ 8-OHdG ng/mg creatinine | −0.04 (3.52) [−1.60, 1.52] | 22 | 0.87 (4.31) [−1.21, 2.95] | 19 |
| Δ Isoprostane ng/mg creatinine | 0.02 (1.53) [−0.65, 0.68] | 23 | −0.06 (1.06) [−0.57, 0.45] | 19 |
| Immune function | | | | |
| Δ PC1 | −0.41 (0.86) [−0.78, −0.04] | 23 | 0.01 (1.21) [−0.60, 0.61] | 18 |
| Δ PC2 | −0.57 (1.15) [−1.06, −0.07] | 23 | −0.44 (1.01) [−0.94, 0.07] | 18 |
| Semen quality | | | | |
| Δ PC1 | 0.20 (0.66) [−0.08, 0.49] | 23 | −0.05 (0.93) [−0.53, 0.43] | 17 |
| Δ PC2 | −0.14 (0.64) [−0.41, 0.14] | 23 | 0.01 (0.57) [−0.29, 0.30] | 17 |
| Δ PC3 | 0.09 (0.75) [−0.25, 0.43] | 21 | 0.52 (0.98) [0.00, 1.04] | 16 |
| Post-supplementation sperm DNA fragmentation % | 11.6 (5.0) [9.1, 13.9] | 22 | 13.9 (8.1) [9.7, 18.0] | 17 |

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