

# What are carotenoids signaling? Immunostimulatory effects of dietary vitamin E, but not of carotenoids, in Iberian green lizards

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**Abstract** In spite that carotenoid-based sexual ornaments are one of the most popular research topics in sexual selection of animals, the antioxidant and immunostimulatory role of carotenoids, presumably signaled by these colorful ornaments, is still controversial. It has been suggested that the function of carotenoids might not be as an antioxidant per se, but that colorful carotenoids may indirectly reflect the levels of nonpigmentary antioxidants, such as melatonin or vitamin E. We experimentally fed male Iberian green lizards (*Lacerta schreiberi*) additional carotenoids or vitamin E alone, or a combination of carotenoids and vitamin E dissolved in soybean oil, whereas a control group only received soybean oil. We examined the effects of the dietary supplementations on phytohaemagglutinin (PHA)-induced skin-swelling immune response and body condition. Lizards that were supplemented with vitamin E alone or a combination of vitamin E and carotenoids had greater immune responses than control lizards, but animals supplemented with carotenoids alone had lower immune responses than lizards supplemented with vitamin E and did not differ from control lizards. These results support the hypothesis that carotenoids in green lizards are not effective as immunostimulants, but that they may be visually signaling the immunostimulatory effects of non-pigmentary vitamin E. In contrast, lizards supplemented with carotenoids alone have higher body condition gains than lizards in the other experimental groups, suggesting that carotenoids may be still important to improve condition.

**Keywords** Body condition · Carotenoids · Immunostimulatory effects · *Lacerta schreiberi* · Lizard · Sexual ornaments · Tocopherol

## Introduction

The evolution of carotenoid-based sexual ornaments has been one of the most popular topics in behavioral and evolutionary ecology in the last 25 years, and several hypotheses have been suggested about the signaling role of carotenoids (reviewed in Olson and Owens 1998; Pérez-Rodríguez 2009; Svensson and Wong 2011). Carotenoids and related metabolites have several important roles in the body such as photoprotection of the retina and the skin (Thomson et al. 2002a, b; Vorobyev 2003; Roberts et al. 2009), a wide range of gene activation and regulatory processes of regeneration, morphogenesis, development, and hormone production (Garbe et al. 1992; Stephensen et al. 2002; Geissmann et al. 2003). Animals are not able to synthesize de novo carotenoid pigments (Goodwin 1984), which have to be acquired from the diet. Carotenoids have antioxidant effects in vitro and may be effective antioxidants in the organism too (Pérez-Rodríguez 2009; Simons et al. 2012). The antioxidant system has very strong relationships with the immune system because macrophages (B and T lymphocytes) and neutrophils produce reactive oxygen species (ROS) when responding to an antigen (Halliwell and Gutteridge 2007) and carotenoids may enhance the immune response by ROS scavenging (Burton 1989; Chew and Park 2004). Thus, carotenoids may have an immunostimulatory effect because of their antioxidant role (von Schantz et al. 1999; Faivre et al. 2003). Therefore, there may be a trade-off between maintaining the antioxidant system and the immune response and elaborating colorful sexual ornaments (von Schantz et al. 1999). This would allow carotenoid-based

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sexual ornaments to be honest signals of health and condition (Lozano 1994; McGraw 2006).

Nevertheless, an alternative hypothesis (Hartley and Kennedy 2004) suggests that the biological role of carotenoids might not be as antioxidants per se, but that colorful carotenoids in sexual signals would just reflect and provide “information” on the true organismal antioxidants, such as the level of melatonin, vitamin C, or vitamin E (Burton and Traber 1990; Brigelius-Flohe and Traber 1999; Martínez et al. 2008), which are, however, not visually informative because they are colorless. This may be explained because oxidation of carotenoids alters or destroys their color (Woodall et al. 1997). Therefore, when a sexual ornament shows carotenoids with their color intensity preserved, it would indicate that there are also other types of efficient colorless antioxidants needed for the protection of color of carotenoids (Hartley and Kennedy 2004). Nevertheless, carotenoids not only have effects on the immune system as antioxidants. They can be involved in the activation of thymocytes (Garbe et al. 1992), the expression of immune-related genes (Geissmann et al. 2003) and the up-regulation of proteins involved in cell-to-cell communication (Basu et al. 2001) and they can increase membrane fluidity (Chew and Park 2004). Therefore, there still may be a trade-off between allocating carotenoids to ornament pigmentation or to the immune response.

There are controversial results on the immunostimulatory effect of carotenoids; several studies have found a positive relationship between carotenoids and some aspects of the immune response in birds (Blount et al. 2003; McGraw and Ardia 2003; McGraw et al. 2006; Aguilera and Amat 2007; Stirnemann et al. 2010) and fish (Amar et al. 2000, 2001, 2004; Clotfelter et al. 2007), but there are also many examples demonstrating a lack of association both in birds (Navara and Hill 2003; McGraw and Ardia 2005; Biard et al. 2006; Hõrak et al. 2006; McGraw and Klasing 2006; McGraw et al. 2006; Sutherland et al. 2012) and fish (Lin et al. 2010). A meta-analysis shows a low effect size for the relationships of circulating carotenoid level with immunocompetence and oxidative stress in birds, suggesting that these relationships might not be causal and that complementary mechanisms maintaining honesty might be involved (Simons et al. 2012). However, studies on the role of carotenoids in other animals also showing carotenoid-dependent coloration, such as some lizards, are less frequent (Olsson et al. 2008; Fitze et al. 2009; López et al. 2009; San-José et al. 2012a, b; Kopena et al. 2014; McCartney et al. 2014), and the relationship between carotenoid intake and immune response has been little explored.

In this study, we examined in the Schreiber’s green lizard (*Lacerta schreiberi*) the immunostimulatory effects of dietary carotenoids in comparison with the effects of a strong, also dietary and lipid-soluble antioxidant, the vitamin E. This lizard is a large sexually dimorphic lacertid from the Iberian Peninsula. Males have, especially during the breeding season,

green dorsal coloration with small black spots, yellow chest, and ventral coloration, and bright blue and UV throat and mental coloration (Martín and López 2009). In contrast, females are mainly dull brown, with pale yellow vents. Inter-individual variation in the characteristics of males’ breeding coloration may signal variation in morphology, health state, dominance, and pairing status (Martín and López 2009). In a previous study, we found that both carotenoids and vitamin E in the diet affect the expression of color ornaments of male *L. schreiberi* lizards. However, in most cases, the addition of vitamin E has a higher effect on the expression of coloration of ornaments than the addition of carotenoids alone, even for those ornaments that are not carotenoid dependent (Kopena et al. 2014).

Here, we experimentally fed male lizards *L. schreiberi* supplementary carotenoids or vitamin E alone, or a combination of carotenoids and vitamin E, dissolved in soybean oil, whereas a control group only received soybean oil. Thereafter, we measured the phytohaemagglutinin (PHA)-induced immune response and the body condition of lizards. We expected that if carotenoids had immunostimulatory effects, lizards supplemented with carotenoids alone would have higher skin-swelling immune responses than control lizards. Moreover, lizards supplemented with vitamin E (alone or combined) would have higher immune responses than control lizards due to the radical scavenger effect of vitamin E (Burton and Traber 1990; Brigelius-Flohe and Traber 1999). Finally, when carotenoids and vitamin E were combined, these would have synergistic effects increasing the immune response. However, if the Hartley and Kennedy’s (2004) hypothesis was true and carotenoids were not true antioxidants in vivo, the immune responses in the carotenoid treatment would not differ from the control one, and the immune responses in the vitamin E and in the combined treatments would be similarly high.

## Materials and methods

### Study animals

In April 2011, we collected 48 adult male lizards *L. schreiberi* from a population inhabiting a pine forest that occupy two contiguous small valleys (“Valle de La Fuenfría” and “Valle de Navalmedio”) (40°44' N, 4°02' W; Cercedilla, Madrid Province, Spain) in the Guadarrama mountains. We immediately transferred lizards to “El Ventorrillo” field station of the Museo Nacional de Ciencias Naturales (Madrid province, Spain), 5 km from the capture site. During all the experiment, lizards were individually kept in outdoor 51 × 36 × 28 cm PVC terraria containing coconut fiber substratum and rocks for cover. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*), house crickets (*Acheta domesticus*), and common

black crickets (*Gryllus assimilis*) dusted with calcium powder, and water was provided ad libitum. At the end of the study, all lizards were released at their exact capture sites in good condition.

We measured immediately after capture and again at the end of the experiment males' body weight with a digital balance to the nearest 0.1 g (initial weight:  $\bar{X} \pm \text{SE} = 26.5 \pm 0.7$  g; range = 18.0–40.5 g) and used a ruler to measure snout-to-vent length, SVL to the nearest 1 mm (initial SVL:  $\bar{X} \pm \text{SE} = 98 \pm 1$  mm; range = 84–110 mm) and tail length ( $\bar{X} \pm \text{SE} = 157 \pm 5$  mm; range = 53–204 mm). Individual values of “body condition” were calculated as the residuals from the regression equation of  $\ln$  mass (in grams) on  $\ln$  SVL (in millimeter), which may represent an index of the relative amount of fat stored and hence an estimation of individual physical condition or nutritional status (Bonnet and Naulleau 1994). Only males ( $N=46$ ) with complete or entirely regenerated tails were considered to estimate body condition.

#### Experimental supplementation of the diet

We selected randomly which individuals were assigned to each of four treatments (“carotenoids”, “vitamin E”, “combination of vitamin E and carotenoids” or “control”; see below). Each male was subjected to daily supplementation for 28 days in his own individual terrarium.

In the “carotenoid” treatment, male lizards were fed daily 8  $\mu\text{g}$  of a mix of a carotenoid complex (Solgar Natural Lutein Lycopene Carotene Complex, purchased from Solgar Inc., Leonia, New Jersey, USA), which contained  $\beta$ -carotene,  $\alpha$ -carotene, zeaxanthin, lutein, and lycopene (in a rate of 5:1.7:0.3:5:5), dissolved in 20  $\mu\text{L}$  soybean oil. Thus, after 28 days of daily supplementation, each male had been provided with a total of 224  $\mu\text{g}$  of carotenoids. The daily amount of carotenoids provided was based on previous studies of carotenoid supplementation in other lizards (Olsson et al. 2008) and corrected for differences in body size between species. Although there is no information on the types of carotenoids found in *L. schreiberi*, studies of another lacertid lizard species reported that lutein and zeaxanthin were the most abundant carotenoids in all tissues, followed by low concentrations of  $\beta$ -carotene (Czeczuga 1980; Fitze et al. 2009; San-José et al. 2012b), all of which were available in our supplementation. This carotenoid profile could result directly from the insectivorous diets of these lizards (e.g., Isaksson and Andersson 2007).

In the “vitamin E” treatment, males were fed daily 20  $\mu\text{L}$  vitamin E supplement (synthetic ( $\pm$ )- $\alpha$ -tocopherol; purchased from Sigma-Aldrich Chemicals Co., St. Louis, Missouri, USA). This contained 97 % of synthetic vitamin E (approximately 1,014 IU  $\text{mL}^{-1}$ ) and 3 % soybean oil (with approximately 0.32 IU  $\text{mL}^{-1}$  of natural vitamin E, i.e., D- $\alpha$ -

tocopherol). Thus, the daily dose provided for each male was approximately 20.2 IU of vitamin E, which is close to the daily minimal physiological necessity of vitamin E for similar-sized reptiles, and well below the tolerable upper intake levels (Mader 1996; Allen et al. 2004; Bender 2009). In other lacertid lizard species,  $\alpha$ -tocopherol was found in abundance in all tissues (San-José et al. 2012b).

In the “combination of carotenoids and vitamin E” treatment, male lizards were fed daily 8  $\mu\text{g}$  of carotenoids and 20  $\mu\text{L}$  of vitamin E as above, both mixed and dissolved together in 20  $\mu\text{L}$  soybean oil. Finally, in the “control” treatment, males were fed daily 20  $\mu\text{L}$  soybean oil alone.

To ensure that all lizards ingested the same amount of carotenoid or vitamin E supplement or the control solution, we gently handled lizards and used sterile plastic syringes with a canula to slowly deliver the solution into their mouth, thus ensuring that lizards swallowed the entire dose.

#### Immune response

One of the most widespread methods for measuring in vivo the immune response is the phytohaemagglutinin (PHA) skin-swelling test (Smits et al. 1999; Kennedy and Nager 2006; de Bellocq et al. 2007; Ardia and Schat 2008). PHA is a plant lectin that induces an artificial activation of the immune system. Although, this test was first referred as an indicator of T-cell-mediated immunocompetence (Hawley et al. 2009; Kilgas et al. 2010), recent studies suggest that the reaction to the PHA injection might be a nonspecific complex inflammation connected with massive infiltration of cells representing both adaptive and innate immunity (Martin et al. 2006; Sarv and Hōrak 2009; Vinkler et al. 2010, 2012; Salaberria et al. 2013). Therefore, the PHA-induced swelling may be a multifaceted index of cutaneous immune activity, and we used this test because we were interested in a standardized index of immunocompetence (Salaberria et al. 2013), independent of the type of immune cells involved.

One day after finishing the diet supplementation procedure, we measured the immune response of lizards by using the PHA injection test (Smits et al. 1999). We used a pressure-sensitive spessimeter to measure thickness (to the nearest 0.01 mm) at the same point of the right hindlimb foot pad before and 24 h after injecting 0.04 mg of PHA dissolved in 0.02 ml of phosphate-buffered saline (PBS) at the marked point. We calculated the immune response as the difference between pre- and postinjection thickness measures (Smits et al. 1999). The only appreciable effect of the PHA injection was a slight swelling of the skin, due to the immune response, which disappeared after 48 h. No lizard showed any sign of stress or pain due to this test.

## Results

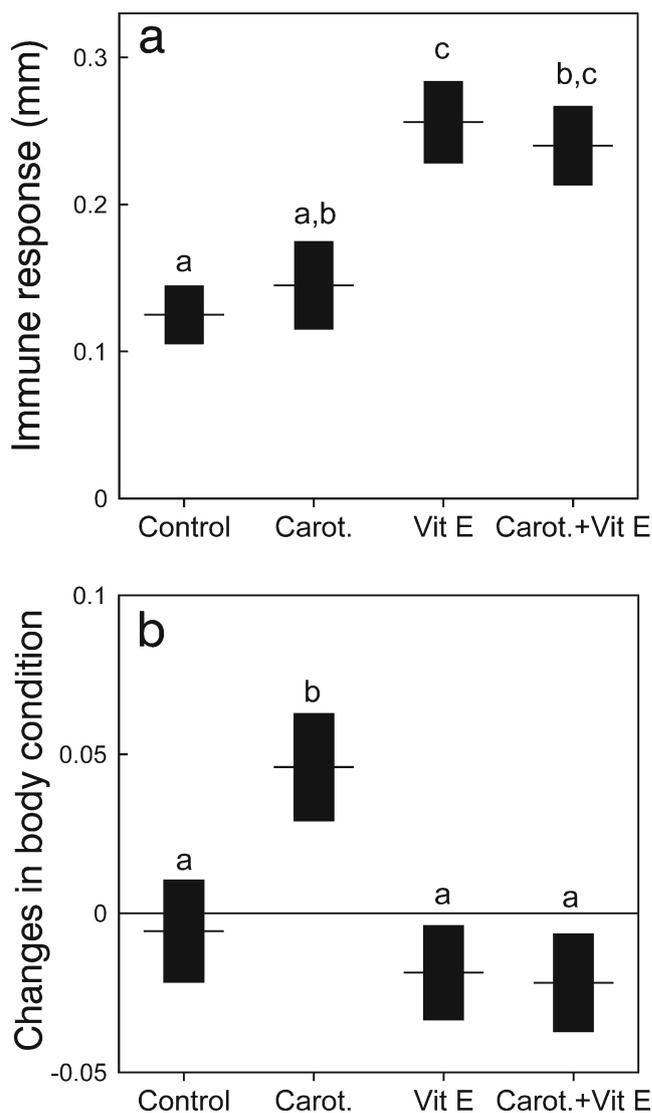
There were significant differences among treatments in the magnitude (log-transformed) of the PHA-induced immune response of lizards after the diet supplementation (one-way ANOVA,  $F_{3,44}=5.74$ ,  $P=0.002$ , Fig. 1). Post hoc tests showed that responses of control lizards did not differ significantly from those of lizards supplemented with carotenoids alone (Tukey's test,  $P=0.98$ ), but had significantly lower responses than lizards supplemented with vitamin E alone ( $P<0.018$ ), or with a combination of vitamin E and carotenoids ( $P<0.01$ ). Lizards supplemented with carotenoids alone had significantly lower responses than lizards supplemented with vitamin E alone ( $P<0.05$ ), but differences between lizards supplemented with carotenoids alone and lizards supplemented with a combination of vitamin E and carotenoids only approached significance ( $P=0.078$ ). Lizards supplemented with vitamin E alone did not significantly differ of lizards supplemented with both vitamin E and carotenoids combined ( $P=0.99$ ).

There were not significant differences among treatments in initial body size (one-way ANOVAs, weight:  $F_{3,44}=0.09$ ,  $P=0.97$ ; SVL:  $F_{3,44}=0.10$ ,  $P=0.96$ ) or initial body condition of lizards ( $F_{3,42}=0.53$ ,  $P=0.66$ ). However, the difference between body condition at the end and at the start of the experiment varied significantly among treatments (one-way ANOVA,  $F_{3,42}=3.76$ ,  $P=0.018$ ). Lizards supplemented with carotenoids alone had positive and significantly greater changes in body condition than lizards supplemented with vitamin E, alone or combined with carotenoids (Tukey's tests,  $P<0.03$  for both) or control lizards ( $P<0.05$ ), all of which had average negative changes in body condition that not differ significantly among them ( $P>0.88$  for all).

The PHA-induced immune response was not significantly related with body condition of lizards at the end of the experiment ( $r=-0.10$ ,  $F_{1,44}=0.53$ ,  $P=0.51$ ) nor with changes in body condition ( $r=-0.04$ ,  $F_{1,44}=0.07$ ,  $P=0.79$ ) or body size (weight,  $r=-0.15$ ,  $F_{1,46}=1.08$ ,  $P=0.30$ ; SVL,  $r=-0.13$ ,  $F_{1,46}=0.83$ ,  $P=0.37$ ).

## Discussion

Our results showed that some of the experimental diet supplementations, but not others, affected the immune response of male *L. schreiberi* lizards. Lizards that were supplemented with vitamin E had heightened PHA skin-swelling immune responses, while the supplementation of carotenoids did not seem to have any effect in comparison with control lizards. Therefore, this experiment may support the hypothesis that carotenoids are not effective immunostimulants, at least in this lizard species and with respect to the aspects of the immune response measured by the PHA test.



**Fig. 1** **a** PHA-induced immune response ( $\bar{X} \pm 1$  SE; in millimeter) and **b** change in body condition ( $\bar{X} \pm 1$  SE) of male lizards *L. schreiberi* in each experimental treatment. Means with the same letter above the bars were not significant different

Contrary to our results, several studies in birds and fish have found a positive effect of dietary carotenoids on the PHA skin-swelling immune response (Blount et al. 2003; McGraw and Ardia 2003; Aguilera and Amat 2007; Clotfelter et al. 2007; Stirnemann et al. 2010), or found that the immune response depletes available carotenoids (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Aguilera and Amat 2007; Pérez-Rodríguez et al. 2008) or found a positive relationship between circulating carotenoid levels and the magnitude of the immune response (McGraw and Ardia 2003; Pérez-Rodríguez et al. 2008). However, many other studies of birds and fish show a lack of effects of carotenoid supplementation on the immune response (Navara and Hill 2003; McGraw and Ardia

2005; Biard et al. 2006; Hörak et al. 2006, 2007; McGraw and Klasing 2006; McGraw et al. 2006; Lin et al. 2010; Sutherland et al. 2012).

We did not find evidence of any effect of carotenoids on the PHA-induced immune response of green lizards. Similarly, carotenoid supplementation has no effects on several aspects of immune performance of growing juvenile chameleons (*Chamaeleo calyptrotus*) (McCartney et al. 2014). In agreement, carotenoid intake in male Australian painted dragon lizards (*Ctenophorus pictus*) does not reduce circulating levels of reactive oxygen species (ROS) or baseline superoxide (bSO), suggesting that carotenoids are inefficient antioxidants in vivo in these lizards. However, the PHA test does not show the whole immune response of the body and therefore we have to narrow our conclusions. Thus, in male society finches, *Lonchura domestica*, supplemented carotenoids do not affect the PHA-induced immune response but increase innate immunity (bacterial-killing activity of whole blood) (McGraw et al. 2006). Similarly, greenfinches, *Carduelis chloris*, with brighter yellow breast feathers show stronger humoral immune response against novel antigen (SRBC) while they do not show any relationship between plumage coloration and the PHA response (Saks et al. 2003). Therefore, carotenoids might still have a role in other aspects of the immune response of green lizards not examined here.

Also, it is possible that just feeding an animal with more antioxidants was not enough to increase directly the antioxidant or immunostimulatory capacity. This is because the levels of different types of antioxidants are homeostatically regulated in animals. Thus, more dietary antioxidants may lead to down-regulation of, for example, antioxidant enzymes in order to maintain some optimal level of antioxidant activity. Further studies might test this possibility with a two-factor study crossing a dietary antioxidant supplement with an antioxidant challenge, which will raise the actual need for additional dietary antioxidants.

Vitamin E supplementation, however, had a clear effect in the heightened PHA-induced immune response of green lizards. Immunostimulatory effects of vitamin E have been repeatedly demonstrated in poultry (Surai 2002). Also, previous studies have shown that green lizards with higher proportions of vitamin E in femoral secretions, which result from higher dietary intake of this vitamin, have heightened immune responses (López and Martín 2006; Kopena et al. 2009, 2011; Martín and López 2010) and are preferred by females (Kopena et al. 2011). This suggests that the cost of allocating antioxidant vitamin E to secretions may confer reliability to chemical signals of green lizards. In contrast, although vitamin E supplementation enhances growth and condition of nestling barn swallows (*Hirundo rustica*), it does not affect their PHA immune response (de Ayala et al. 2006). Similarly, in greenfinches (*C. chloris*), there is no effect of vitamin E on the PHA reaction. However, this could be explained because

birds in all treatments were fed sunflower seeds, which are one of the richest seeds in natural vitamin E content and this could mask the differences between the vitamin E supplemented and the control groups (Hörak et al. 2007). In a similar study, supplementary vitamin E had no effect on the PHA skin-swelling response of 21-week-old ring-necked pheasants (*Phasianus colchicus*), but there were 13 weeks between the vitamin E dietary supplementation and the PHA test and probably pheasants did not store vitamin E to be used as antioxidant but allocated it into development (Orledge et al. 2012).

Lizards supplemented with carotenoids plus vitamin E combined had greater immune responses than control lizards, but these responses only tended to differ marginally of lizards supplemented with carotenoids alone. This is an interesting result, because these two antioxidants may have synergistic effects (Leibovitz et al. 1990; Palozza and Krinsky 1992; Surai et al. 2001). It is possible that increasing carotenoids intake required using part of the supplemented vitamin E to protect these carotenoids from oxidation in colorful traits, and, thus, not all vitamin E could be used in other antioxidant functions. This would result in that lizards supplemented with vitamin E alone would actually have more vitamin E available to be used in antioxidant functions related to the immune response, leading to greater differences between treatments. Another possible reason is that the mechanism of carotenoid absorption in the intestine was similar to that of vitamin E (Woodall et al. 1996; Surai 2002). Therefore, increased carotenoid supplementation might have induced competitive interactions between carotenoids and vitamin E during absorption, and supplemented carotenoids might have impaired the intestinal absorption of vitamin E, as reported in humans (Reboul et al. 2007).

Finally, body condition of lizards supplemented with carotenoids alone increased, while lizards supplemented with vitamin E or with carotenoids combined with vitamin E had decreased body condition. This suggests that carotenoids may have some important role affecting positively to health state and condition of lizards, possibly as antioxidants. However, changes in body condition were not related to differences in the immune response. In some growing nestlings bird species, carotenoid supplementation may also increase body condition by regulating oxidative stress resulting from rapid growth (Biard et al. 2006), although in other fish and bird species there are no effects (e.g., Pike et al. 2010; Sutherland et al. 2012), probably because natural food might contain sufficient carotenoids to obscure any benefit of carotenoid supplementation. A similar positive effect of carotenoids on body condition might occur in lizards which have continuous growth and, in our captivity experiment, only have access to some prey types that might not contain enough natural carotenoids. However, supplementary carotenoids did not increase body weight of captive common lizards, *Lacerta vivipara* (San-José

et al. 2012a). Interestingly, the positive effect of carotenoids disappeared when carotenoids were given together with vitamin E, suggesting that dietary vitamin E and carotenoids may interact with each other in a complicated fashion.

In summary, our previous research examining the effects of antioxidants on sexual signals of *L. schreiberi* (Kopena et al. 2014) and the present results support the hypothesis that carotenoids are not effective as immunostimulants, but that they may be visually signaling the immunostimulatory effects of non-pigmentary vitamin E. However, we have to interpret carefully the results of the PHA test as indicator of immunocompetence (Kennedy and Nager 2006) and for clearer conclusions we need further experiments that examine other aspects of the antioxidant and immune system of lizards. Also, the possible synergistic or competitive effects of carotenoids and vitamin E should be examined.

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**Ethical standards** The experiments enforced all the present Spanish laws and were performed under license (permit number: 10/142790.9/11) from the Environmental Organisms of Madrid Community where they were carried out.

**Conflict of interest** None

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