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Influences of carotenoid supplementation on the integrated antioxidant system of a free living endangered passerine, the hihi (*Notiomystis cincta*)

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Abstract

The integrated antioxidant system is recognised as an essential component of an organisms self maintenance. Our knowledge of this system, however, is largely restricted to species of economic importance. The health and productivity benefits these dietary based compounds provide make them increasingly relevant for study in wildlife ecology. The aim of this research was to identify numerous components of this integrated system in a free living and endangered passerine bird, the hihi. In addition experimental supplementation with carotenoids was used to investigate the modulatory interactions with other members of the antioxidant system. Our results identified lutein and zeaxanthin as the carotenoids utilised by hihi (82% and 17% of total carotenoids respectively in control samples of egg yolk, 84% and 16% of total carotenoids respectively in control samples of nestling plasma), and that vitamin E was represented by both α- and γ-tocopherol. Retinol was also present, as was selenium in surprisingly high concentrations (599.64, 91.76, 377.72 ng/g fresh weight Se in control samples of yolk, albumin and plasma, respectively). Supplementation of lutein and zeaxanthin not only increased their presence in egg yolk ($F_{1,10}$=14.285, P=0.005 and $F_{1,10}$=9.606, P=0.015, respectively) and nestling plasma ($F_{1,19}$=35.126, P<0.001 and $F_{1,19}$=28.597, P<0001, respectively) but also led to increased selenium concentration in egg yolk ($F_{1,10}$=7.213, P=0.028), increased retinol concentration in nestling plasma ($F_{1,19}$=4.272, P=0.054) and decreased α-tocopherol concentration in nestling plasma ($F_{1,19}$=5.122, P=0.037). These results provide detail of the antioxidant system in novel taxa and importantly highlight interaction between these various compounds. Given their increased application in productivity and health in agriculture and human medicine we highlight the potential application of this knowledge in wildlife ecology and conservation.

Keywords: Antioxidant; Carotenoids; Conservation; Egg yolk; Plasma; Retinol; Selenium; Vitamin E

1. Introduction

All living organisms are under constant attack from free radicals. In particular, superoxide is considered the major free radical stress produced by living cells (Halliwell and Gutteridge, 1999). Its production comes as the unavoidable by-product of metabolism, and as part of the immune systems defence against foreign micro-organisms (Schwarz, 1996; Surai, 2002). To survive individuals need protection from these, and other, radical species. Radical species are known to damage lipids, proteins and DNA and, as a consequence, affect membrane composition and structure, enzymatic activities and cause mutagenesis. As such a delicate balance is required between the sometimes necessary presence of radicals and a protective system to control them. The integrated antioxidant system is the collective term used to describe the varied compounds involved in cell protection. This system includes the fat soluble antioxidants (including vitamins A and E, coenzyme Q and carotenoids), water soluble antioxidants (including ascorbic acid, uric acid), antioxidant enzymes (including glutathione peroxidase (GSH-Px), superoxide dismutase and catalase) and the thiol redox system (including both the glutathione and thioredoxin systems) (Surai, 2002).

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Although each antioxidant has specific roles in this integrated system there is also a level of interaction and modulation between these different compounds. For example, carotenoids are known to be precursors of vitamin A, being converted to retinoids in the intestinal wall (Cheng and Deuel, 1950). However, less than 10% of known carotenoids are converted into vitamin A and the role of more than 500 additional carotenoids awaits investigation (Surai, 2002). Interactions between carotenoids and vitamin E are not clear yet. Numerous studies have shown positive, negative, and no effects of carotenoids on vitamin E levels (discussed in Surai, 2002). Further, synergistic interactions have also been shown. For example, providing lycopene (a red carotenoid) to female rats increased the activities of antioxidant enzymes such as GSH-Px (Breinholt et al., 2000). These interactions depend somewhat on which compounds are utilised within each antioxidant family. Each species has its own combination of these compounds, governed by a mix of dietary availability and phylogeny (e.g., in carotenoids see Tella et al., 2004), making it difficult to determine the optimal antioxidant balance of any given taxa.

Our knowledge is largely restricted to model laboratory species (such as rats and mice), humans and domestic animals. Very little information on antioxidant systems is available in non-domestic and free living wild species. This is somewhat surprising given the growing interest in the fitness advantages antioxidants accrue to individuals (e.g., see Alonso-Alvarez et al., 2004; Blount et al., 2004, 2003; McGraw and Ardia, 2003). Carotenoids in particular are the focus of extensive ecological and evolutionary research due primarily to their dual function in self maintenance and as pigments in animal ornamentation (Moller et al., 2000). The clear health advantages and dietary basis of carotenoids has also resulted in an application to measurements of environmental stress (Eeva et al., 1998; Horak et al., 2000). Unfortunately, most research in this area does not consider the interactions of carotenoids with other antioxidants despite the obvious potential to extend our knowledge of integrated antioxidant systems to varied taxa. In addition, there is obvious potential to apply this knowledge, along with lessons learnt in domestic animal production, to address questions in wildlife health.

The aims of our study were therefore twofold. Firstly, to document the concentrations of various antioxidants in a free living but endangered bird species, and secondly to investigate the effects of supplementing carotenoids on other components of their integrated antioxidant system. We fed breeding hihi (*Notiomystis cincta*) carotenoids known to be utilised by this species. Our first question was to determine whether carotenoids supplemented to breeding females were deposited into egg yolk and whether this, in addition to direct provisioning from parents, resulted in higher carotenoid concentration circulating in nestling plasma. Secondly, we wanted to investigate the effects of this increased carotenoid availability on various additional antioxidants in both egg and plasma samples. Our focus was on the fat soluble antioxidants, vitamins A and E, and Selenium (Se) which is an important component of the antioxidant enzyme glutathione peroxidase (GSH-Px).

2. Materials and methods

The hihi (or stitchbird, *Notiomystis cincta*) is an endangered passerine endemic to New Zealand whose diet consists of nectar, fruit and invertebrates. Although once widespread throughout the North Island of New Zealand the species suffered a rapid decline following European colonisation and by 1890 had become restricted to a single offshore island population (3083 ha. Hauturu). Conservation of this species relies on reintroducing birds to additional island and mainland habitats and has met with limited success (Taylor et al., 2005). One hypothesis for this poor success is pathogen stress on immunocompromised individuals (Alley et al., 1999; Taylor et al., 2005). This study was conducted on Tiritiri Matangi Island, the single reintroduced population (established in 1995) which shows positive population growth.

Previous research has identified lutein and zeaxanthin as the carotenoids circulating in adult hihi plasma, and used as pigments for the males bright plumage (82% and 14%, respectively, Ewen et al. unpublished data). We therefore provisioned these carotenoids in the form of a commercially available product OroGLO® supplied by Kemin Industries. OroGLO® comprises 82.69% *trans*-lutein and 6.07% *trans*-zeaxanthin which provides the approximate ratio of these carotenoids as found in hihi plasma. Hihi on Tiritiri Matangi are provisioned with sugar water (20% by mass) for management and we took advantage of this to conduct our carotenoid supplementation feeding experiment. All breeding pairs were individually provided with supplementary food (placed within 10 m of the nest) from the commencement of nest building until nestlings fledged (at age 30 days). Only nests with simple pairs (i.e., one social male and one female parent) were included in the study. Female age did not vary between treatment groups ($F_{1,46}=2.73$, $P=0.11$) and no obvious health differences were detectable between females in this population. In half these nests carotenoids were added to sugar water to a final concentration of 100 μg/mL following results from immune activation and antioxidant activity to variable dose rates of OroGLO® in the zebra finch (Alonso-Alvarez et al., 2004). Carotenoids were provided in brown plastic bottles to avoid light and all feeders were changed every second day to keep food fresh. A sample of nests was observed for one hour each to determine if supplementary food was taken. There was no significant difference in the use of these feeders between treatment groups (carotenoid $N=11$, mean female visits/hour $4.5=SE=0.45$; sugar $N=7$, mean female visits/hour $3.7=SE=0.57$; $F_{1,16}=1.04$, $P=0.32$). Nests were checked daily following the commencement of egg hatching and all un-hatched eggs were collected three days after their expected hatch date (14 days after incubation starts). Any unfertilised eggs were separated into their yolk and albumen components and stored frozen until antioxidant analyses. Surviving nestlings had blood samples collected at 24 days old. Blood was immediately centrifuged and plasma removed and similarly stored frozen until antioxidant analysis.

Carotenoids and vitamins A and E were determined by high performance liquid chromatography (HPLC) as described

Table 1
Mean concentration (μg/g) of fat soluble antioxidants in unfertilised egg yolk of hihi (n=5 sugar and 5 carotenoid egg yolks each from a different female)

Antioxidant	Treatment	Concentration (μg/g)	Standard error
Total carotenoids*	Sugar	26.69	4.9
	Carotenoid	182.55	42.71
Lutein*	Sugar	21.74	3.92
	Carotenoid	143.59	31.95
Zeaxanthin*	Sugar	4.78	1.29
	Carotenoid	38.8	10.88
Retinol	Sugar	1.91	0.25
	Carotenoid	1.88	0.45
9-tocopherol	Sugar	35.34	8.81
	Carotenoids	26.00	3.87
γ-tocopherol	Sugar	1.39	0.4
	Carotenoid	2.41	0.26

* Denotes significant treatment effects with $P<0.05$.

elsewhere (see Cassey et al., 2005 for carotenoids and Karadas et al., 2005 for vitamin A and E). In brief, egg yolk (100–200 mg) was homogenised with 0.7 mL NaCl 5% and 1 mL ethanol, and carotenoids were extracted by adding 2 mL hexane with further homogenisation. The hexane phase was then collected following centrifugation (extractions were repeated twice). Procedures were similar for plasma samples with the exception that 40 μL of plasma was vortexed with 1 mL of ethanol before the double hexane extraction (with 400 μL hexane). Hexane extracts were pooled and evaporated at 60–65 °C under nitrogen flow, and the residue was dissolved in 0.1 mL dichloromethane and 0.1 mL methanol. Individual carotenoids were detected with a Spherisorb type ODS2 5 μ C18 reverse-phase column, 25 cm × 4.6 mm (Phase separation, Clwyd, UK) with a mobile phase of acetonitrile-methanol (85 : 15) and acetonitrile–dichloromethane-methanol (70 : 20 : 10) at a flow rate of 2 mL min^{-1}, using detection by absorbance at 445 nm. Vitamin A (retinol) and vitamin E (α- and γ-tocopherol) were detected with a Spherisorb type S30DS2 3 μ C18 reverse phase column, 15 cm × 4.6 mm (Phase separation, Clwyd, UK) with a mobile phase of methanol/water (97 : 3, v/v) at a flow rate of 1.05 mL min^{-1}, using detection by excitation at 295 nm and emission at 330 nm. Standard solutions of α- and γ-tocopherol in methanol were used for calibration and tocol was used as an internal standard. Selenium concentrations were determined as detailed in (Pappas et al., 2005). This technique utilised hydride generation atomic fluorescence spectroscopy of nitric (HNO_3): water (H_2O): perchloric acid ($HClO_4$): sulphuric acid (H_2SO_4), 50 : 17.4 : 25 : 7.6, v/v/v/v digested samples. Selenium in the subsequent residue was dissolved in 3 M hydrochloric acid and any selenate was converted to selenite by gentle heating. The method used a hydride generator, a fluorescence detector (Model 10.033, PS Analytical Ltd, Kent, UK) fitted with a boosted discharge hollow cathode lamp (superlamp Se, Photon, PTY Ltd, Australia) an autosampler (Model 20·099, PS Analytical Ltd, Kent, UK) and Avalon ™ (PS Analytical Ltd, Kent, UK) software.

In total, five yolk samples from each treatment were available for carotenoid and vitamin analysis (each coming from a different breeding female). Ten plasma samples per treatment (one per breeding female) were also analysed for their carotenoid and vitamin concentrations. An additional five yolk, albumin and plasma samples per treatment were available for Se analysis (similarly from independent breeding females). These samples were different from those used for carotenoid and vitamin analysis because of the amount of yolk and plasma required to determine Se concentration. Statistical analyses were conducted in SPSS 12.0.1. A mix of general linear models (GLMs) and ANOVA were used to investigate differences in the concentration of each antioxidant between yolk, albumin and yolk along with treatment (sugar versus carotenoid supplemented). As sugar water is permanently available to hihi, we regard our sugar treatment as a control and interpret antioxidant concentrations in this group as representative of their natural levels. Descriptive statistics are means ± SE.

3. Results

Five egg yolk samples were selected from each treatment (each from a different female) to determine their carotenoid and vitamin concentrations. As previously shown in adult hihi plasma (see Methods), the carotenoids utilised by hihi are lutein and zeaxanthin (making up 82% and 17% respectively of egg yolk carotenoids; Table 1). No other carotenoids were detected. The other fat soluble antioxidant present in high concentrations was α-tocopherol, which contrasted with the relatively low concentrations of both retinol and γ-tocopherol (Table 1). Eggs from supplemented females had significantly higher concentrations of both lutein and zeaxanthin (Table 1; $F_{1,10}=14.29$, $P=0.005$ and $F_{1,10}=9.606$, $P=0.015$, respectively). There was little difference in either the concentration of retinol ($F_{1,10}=0.01$, $P=0.947$) or α-tocopherol ($F_{1,10}=0.94$, $P=0.361$) between treatments, however, there was a near significant treatment effect on γ-tocopherol, with a tendency for higher levels in eggs from carotenoid supplemented females (Table 1; $F_{1,10}=4.03$, $P=0.079$).

To investigate whether these, or any other differences, were present in the circulating antioxidants of nestling birds we

Table 2
Mean concentration (μg/g) of fat soluble antioxidants in nestling plasma (n=10 sugar and 9 carotenoid samples each from a different nest and female)

Antioxidant	Treatment	Concentration (μg/g)	Standard error
Total carotenoids**	Sugar	16.64	4.75
	Carotenoids	94.44	12.9
Lutein**	Sugar	13.96	3.05
	Carotenoid	84.08	7.98
Zeaxanthin**	Sugar	2.68	0.4
	Carotenoid	10.36	0.96
Retinol*	Sugar	0.30	0.04
	Carotenoid	0.47	0.04
9-tocopherol**	Sugar	5.64	0.46
	Carotenoids	3.28	0.58
γ-tocopherol	Sugar	1.19	0.2
	Carotenoid	1.47	0.3

** Denotes significant treatment effects with $P<0.05$, * denotes near significant treatment effect with $P=0.054$.

Table 3
Mean concentration (ng/g fresh weight) of selenium in different sample types of hihi ($n=5$ each of egg yolk, egg albumen and nestling plasma)

Sample type	Treatment	Concentration (ng/g)	Standard error
Egg yolk*	Sugar	599.64	56.6
	Carotenoid	862.49	39.92
Egg albumen	Sugar	91.76	11.27
	Carotenoid	105.34	6.69
Plasma	Sugar	377.72	21.07
	Carotenoid	330.88	32.04

* Denotes significant effect of treatment on selenium concentration with $P<0.05$.

analysed twenty plasma samples (10 from each treatment). One sample, however, was lost due to a problem with the HPLC. Similar to the patterns seen in egg yolk, the major fat soluble antioxidants detected in plasma were the carotenoids lutein and zeaxanthin along with high levels of α-tocopherol and only small amounts of retinol and γ-tocopherol (Table 2). There was a significant decrease in the concentration of both retinol ($F_{1,15}=32.99$, $P<0.001$) and α-tocopherol ($F_{1,15}=23.99$, $P<0.001$) between egg yolks and plasma in control samples. No differences were detected with the other antioxidants measured (lutein, zeaxanthin and γ-tocopherol).

Nestlings from carotenoid supplemented parents had significantly higher lutein and zeaxanthin concentrations (Table 2; $F_{1,19}=35.13$, $P<0.001$ and $F_{1,19}=28.6$, $P<0001$, respectively). There was also a significant effect of treatment on α-tocopherol ($F_{1,19}=5.12$, $P=0.037$) and a near significant effect of treatment on retinol ($F_{1,19}=4.27$, $P=0.054$). Interestingly, these were in opposite directions, with a decrease in α-tocopherol concentration in carotenoid supplemented individuals and an increase in retinol concentration (Table 2). No effect of treatment on γ-tocopherol was observed ($F_{1,19}=0.32$, $P=0.578$).

Finally, five additional samples each of egg yolk, egg albumen and plasma were analysed for their Se content. There was a highly significant effect of sample type (yolk, albumin, plasma) on Se levels ($F_{2,15}=95.52$, $P<0.001$), with the highest concentrations found in egg yolk and the lowest in egg albumen (Table 3). Although experimental treatment was not significant in this model ($F_{1,15}=2.6$, $P=0.12$) there was a significant interaction with sample type ($F_{2,15}=7.81$, $P=0.002$). Further investigation revealed this was the result of a significant treatment effect in egg yolk only, with higher Se concentrations in carotenoid supplemented eggs (Table 3; $F_{1,10}=7.21$, $P=0.028$).

4. Discussion

Our study has identified the fat soluble antioxidants utilised by hihi and quantified the levels of Se in both eggs and nestling plasma. As expected lutein and zeaxanthin are the carotenoids deposited in egg yolk and circulating in plasma. Vitamin E was represented predominantly by α-tocopherol with additional small amounts of γ-tocopherol. Both retinol and α-tocopherol concentration decreased between samples of egg yolk and plasma. No differences were noted in carotenoids or γ-tocopherol. Importantly, our supplementation experiment succeeded in increasing the concentration of both lutein and zeaxanthin. In addition, our supplementation resulted in increases in Se (egg yolk) and retinol (nestling plasma) and a decrease in α-tocopherol (nestling plasma).

Total carotenoid concentration in hihi egg yolk was most similar to samples of other passerine species such as the European starling, *Sturnus vulgaris* (Cassey et al., 2005), and the blue tit, *Parus caeruleus* (Biard et al., in press) along with feral Canada geese, *Branta canadensis* (Speake et al., 1999). These concentrations are higher than those typically reported for captive domestic and non-domestic species (Karadas et al., 2005; Speake et al., 1999). Compared to a much broader range of free living species, total carotenoid concentration in hihi plasma appeared to be relatively high (Tella et al., 2004). This is expected given comparative analyses suggest species utilising carotenoids in ornamentation circulate increased levels of these compounds (Cassey et al., 2005; Tella et al., 2004). Levels of α-tocopherol in egg yolk were similar to those reported in free range chicken, *Gallus domesticus* and guinea fowl, *Numida meleagris* (Karadas et al., 2005) and in free range domestic geese, *Anser anser domesticus* (Speake et al., 1999). Although substantially lower than levels reported in seabirds (Surai, 1999; Surai et al., 2001a,b), α-tocopherol concentration in hihi egg yolk was still higher than reported in some captive species (Karadas et al., 2005; Speake et al., 1999); but note higher concentrations reported in captive chickens supplemented with synthetic vitamin E (Karadas et al., 2005; Surai, 1999). Plasma α-tocopherol concentrations in hihi were similar to those reported in commercial hen samples (Surai, 1999). Both γ-tocopherol and retinol concentrations were low in hihi egg yolk and plasma. This seems to agree with γ-tocopherol levels reported in both wild and captive ducks, chickens and guinea fowl (Karadas et al., 2005) and again is lower than reports of seabirds (Surai et al., 2001a,b). Species differences in retinol requirements are less pronounced but follow similar patterns with seabirds characterised by higher concentrations (Surai et al., 2001a,b) and another passerine, the blue tit having similar concentrations in egg yolk (Biard et al., in press).

Selenium availability in the New Zealand environment is regarded as naturally low and has resulted in extensive supplementation in agriculture (Thomson, 1989; Wells, 1967). Despite this, New Zealanders have been reported to have levels below those recommended for efficient function of antioxidant enzymes such as GSH-Px (Duffield, 1999). Interestingly, our results show hihi had high concentrations of Se in all sample types, being substantially higher in egg yolk and nestling plasma than in egg albumen. These levels are consistent with the high levels reported in egg yolk from another endangered species found on Tiritiri Matangi Island, the Takahe *Porphyrio hochstetteri* (Jamieson and Ryan, 2001). Takahe sampled from other localities in New Zealand had relatively lower Se concentrations (Jamieson and Ryan, 2001), raising the possibility that Tiritiri Matangi has high environmental availability of this element. The Se concentrations found in hihi egg yolks are similar to those reported in egg yolks of chickens fed a diet supplemented with organic selenium at 0.2 mg/kg (Surai, 2000).

Supplementation of carotenoids had no effect on retinol or tocopherol concentrations in egg yolk of hihi. This mirrors results of a recent supplementation experiment in blue tits (Biard et al., in press). Carotenoid supplementation did, however, cause a decrease in the concentration of α-tocopherol in nestling plasma. Similar patterns have been found in chicken plasma (after supplementation with β-carotene, Lim et al., 1992; Woodall et al., 1996), and astaxanthin (Lim et al., 1992). We also found an almost significant increase in retinol concentration in nestling plasma. This is not immediately intuitive given the carotenoids absorbed by hihi are not renowned for the provitamin A activity (Surai, 2002). However, both lutein and zeaxanthin have been shown to have provitamin A activity in some circumstances, namely retinol deficiency (Hencken, 1992; Weiser and Kormann, 1993). When considering the concentration of vitamins A and E in plasma it is necessary to take into account the balance of these vitamins in the body. Indeed the liver is considered to be the storage organ for vitamin A and an important organ for vitamin E metabolism. Therefore, changes in concentration of these vitamins in nestlings could reflect their redistribution in the body. In particular, higher carotenoid concentration in plasma would build increased antioxidant defences and therefore reduce the requirement for vitamin E in self-maintenance thus lowering its concentration in plasma. There is a possibility that more vitamin A was released from the liver as a result of carotenoid dietary supplementation. However, these explanations need further clarification in future experiments.

Surprisingly, Se was also shown to increase in hihi egg yolk of carotenoid supplemented females. No other study, known to the authors, has investigated the effects of carotenoid supplementation on Se levels. Based on present knowledge it is difficult to explain this phenomena. Indeed, Se absorption from the feed is dependent on the integrity of the digestive tract, in particular enterocytes. Recently, it has been suggested that antioxidant–prooxidant balance in the digestive tract is a key for general health (Surai et al., 2003, 2004) therefore, it could well be that carotenoid supplementation improves the balance in the digestive tract and facilitates better Se absorption. Se supplementation, however, appears to have no effect on carotenoid concentration, yet does increase vitamin E concentration in both egg yolk and plasma of domestic chickens (Surai, 2000). There are other possible explanations for the observed differences in Se concentration. Either females receiving carotenoids foraged differently to control females (it could well be that because of carotenoid supplementation they use different foods which could be lower in carotenoids but higher in Se), and/or females with increased antioxidant resources (in the form of carotenoids) were able to differentially invest Se into their eggs. Further research is necessary to test these ideas.

The antioxidant abilities of carotenoids, vitamins A and E, and selenium are well known (reviewed in Surai, 2002). In addition these compounds provide numerous non-antioxidant advantages. Carotenoids are widely used as pigments in animal signaling and are involved in efficient immune activation (Moller et al., 2000). Vitamin E and Se deficiencies are related with a range of pathological conditions yet, these same compounds can be detrimental if present in excess (similarly reviewed in Surai, 2002). The numerous roles these compounds play, and their interactions, present a highly complex system of self maintenance within an individual. The investment decisions of females in reproduction is thus of extreme importance to the improved fitness of themselves and their offspring. This has been a major focus of agricultural research for economic reasons (Surai, 2002). In the case of the hihi, this is of direct application to conservation management. Importantly, we now have an improved understanding of the nutrients utilized by this species. Further research is currently investigating both the dietary availability of these nutrients (between habitats of varying disturbance) and experimentally testing the health advantages of supplementing components of the antioxidant system (J.Ewen unpublished data).

Understanding the diversity of integrated antioxidant systems requires investigating and manipulating a range of taxa beyond those model species of commercial importance. Not only is this of fundamental interest to biochemists, but is of growing importance in fields of ecology with an interest in testing adaptive theories of evolution. Interpretation of results is currently hindered by a lack of knowledge regarding both modulation and synergistic effects between different antioxidant compounds. Further, there is potential to use this information to understand and alter decreased productivity and viability in wildlife under growing environmental stress.

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