TECHNICAL COMMENT

Repeated Sampling but Not Sampling Hour Affects Plasma Carotenoid Levels

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ABSTRACT

Carotenoid pigments have become a central subject of research on animal signaling systems during the past decade. Thus, measurement of plasma carotenoid levels is widespread in the literature. Many plasma biochemical parameters tend to vary with the hour of sampling, which may be an important source of sampling error. However, little is known about this kind of variation for circulating carotenoids. With a sampling protocol that allowed us to separately analyze the effect of sampling hour and repeated blood extraction at the within-individual level, we evaluated the effect of these two parameters on plasma carotenoid concentration in captive red-legged partridges (*Alectoris rufa*). The hour of sampling did not show a significant influence on carotenoid variability. Nevertheless, carotenoid levels significantly decreased as a result of repeated sampling. In fact, carotenoid variability was positively related to hematocrit values, which also decreased throughout the experiment. Furthermore, the effect was evident from the second sampling event. These results suggest that blood samples for carotenoid analysis may be obtained at random during the daytime with no serious risk of adding variance to data obtained. However, the effect of hemodilution associated with repeated blood extraction should be carefully considered in those studies involving repeated sampling.

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Introduction

Carotenoid pigments are currently the focus of interest of behavioral and evolutionary ecologists because of their role as the biochemical basis for most yellow, orange, and red colors in animals (Goodwin 1984). Because carotenoids cannot be synthesized by vertebrates de novo but must be obtained from the diet, it has long been argued that carotenoid-based colors function as reliable signals of the foraging ability of individuals, particularly birds and fishes (Olson and Owens 1998; Hill et al. 2002). However, within the past decade, it has also been suggested that these carotenoid-derived sexual colors may signal the health status of individuals as a result of their antioxidant and immunostimulatory activity (Lozano 1994; von Schantz et al. 1999; Møller et al. 2000; McGraw and Ardia 2003).

This great interest in carotenoids as a key factor within the sexual and social honest signaling context has led in the past years to an exponential increase of empirical studies in which plasma carotenoid levels are measured and related to signal development (Saino et al. 1999; Blount et al. 2003; Peters et al. 2004), parasite load (Allen 1992), immunocompetence (Saino et al. 1999; Blount et al. 2003; McGraw and Ardia 2003), and antioxidant capacity (Alonso-Alvarez et al. 2004; Bertrand et al. 2006). However, it is known that many blood parameters show daily patterns of variation that may reduce the reliability of the data that have been obtained (Wisser and Breuer 1981; García-Rodríguez et al. 1987; Ferrer et al. 1994). The assessment and control of this source of within-individual variation is essential for the development of correct and accurate sampling procedures, thus avoiding undesirable sources of variation.

It is known that blood plasma carotenoid levels in birds may be affected by diet (Negro et al. 2000; McGraw et al. 2003) and covaries with age (Negro et al. 2001) and sex (Bortolotti et al. 1996; McGraw et al. 2003). To our knowledge, only one study (Hõrak et al. 2004) has described the possible daily variation in avian plasma carotenoid levels, under field conditions and at the interindividual level, finding a significant positive correlation with the hour of sampling that had to be controlled for in the analyses by means of statistical procedures (ANCOVA). The existence of such daily patterns of variation in plasma carotenoids demands further study and the design of a specific experimental protocol to evaluate under controlled conditions (i.e., constant diet composition and repeated sampling of the same individuals) the real relevance of sampling

Table 1: Results of a general linear mixed model for plasma carotenoid levels as dependent variable

	Plasma Carotenoids		
Fixed Factors	*F*	df	*P*
Sex	.42	1, 26	.52
Hour of sampling	1.53	3, 74	.21
Day of sampling	2.65	3, 74	.055
Hematocrit	14.64	1, 74	.0003

Note. Sex, day, and hour of sampling were entered as fixed factors and hematocrit as covariate. Individual identity was entered as a random variable ($Z = 2.98$, $P = 0.003$), thus controlling for the repeated sampling effect. No interaction was significant (all $P > 0.10$), therefore being excluded from the final model.

hour as a source of bias in our results. This knowledge would contribute to design sampling procedures that avoid those time lapses where most circadian variability is present (e.g., avoiding samplings close to sunrise and sunset).

In this study, we assessed experimentally whether a daily variation in plasma carotenoid levels exists. The study species, the red-legged partridge (*Alectoris rufa*), is a midsize galliform displaying red coloration in bill, legs, and eye lores, which is related to plasma carotenoid levels (Blas et al. 2006). Plasma carotenoids in the red-legged partridge vary with age, sex, and season, even when diet composition is held constant (Negro et al. 2001). Therefore, from a physiological and evolutionary perspective, the red-legged partridge is a good model for the study of carotenoid-based ornaments.

Methods

The experiment was performed at the Dehesa Galiana experimental facilities (Instituto de Investigación de Recursos Cinegéticos, Ciudad Real, central Spain) during April 2006. At this period of the year, both sexes show their maximum seasonal levels of plasma carotenoids (L. Pérez-Rodríguez, unpublished data). We randomly selected for the study 14 male and 14 female 4-yr-old red-legged partridges hatched in captivity. These birds came from a governmental breeding facility placed at Lugar Nuevo (Andujar, Córdoba, Spain; Bortolotti et al. 2003), a population obtained from wild birds captured in a neighboring hunting reserve. Birds were housed in individual outdoor cages (1 m × 0.5 m × 0.4 m) at ambient temperature and natural photoperiod (13L : 11D). Individuals were fed with commercial pelleted food (20% protein, 4.5% fat, 3.7% cellulose) containing 5.26 μg of carotenoids (96% lutein) per gram of food.

Each bird was sampled once a day at 0815, 1215, 1630, and 2030 hours (just at sunset) on alternate days (April 6, 8, 10, and 12; days 1–4, respectively, hereafter). Sunrise takes place at 0720 hours at this time of year. Therefore, birds had approximately 1 h to break their fast before the first sampling. Therefore, the study lasted 6 d and involved four samplings per bird. However, in order to distinguish between circadian variability and the effect of consecutive sampling (day effect), sampling hour was randomized at the individual level (e.g., bird A: day 1, 1215 hours; day 2, 2030 hours; day 3, 0815 hours; day 4, 1630 hours; and so on). One of the females escaped before the last blood sample was collected, so sample size is reduced by one individual in the statistical analysis.

In all cases, 500 μL of blood was collected from the brachial vein in heparinized syringes. For each blood sampling, birds were extracted from the cage and handled for fewer than 2 min each. A 75-μL heparinized capillary was filled from the sample in order to measure the hematocrit, which would allow us to detect a possible hemodilution caused by repeated sampling. Blood samples and capillary tubes were kept cold until centrifugation (no more than 3 h later) at 10,000 rpm for 8 min. Plasma was stored at −80°C until analysis.

Carotenoids were quantified by diluting 60 μL of plasma in acetone (1 : 10). The mixture was vortexed and centrifuged at

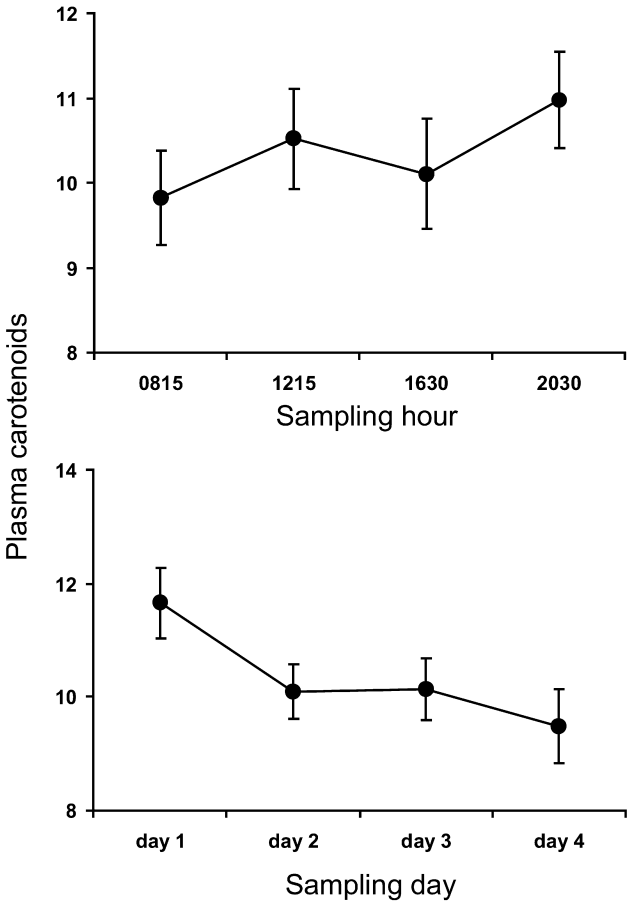

Figure 1. Plasma carotenoid levels (μg/mL plasma) in relation to sampling hour (*top*) and day of sampling (*bottom*) in red-legged partridges. Values are means ± SE.

Table 2: Results of a general linear mixed model for hematocrit variability as dependent variable

	Hematocrit		
Fixed Factors	*F*	df	*P*
Hour of sampling	.28	3, 75	.84
Day of sampling	7.39	3, 75	<.001
Sex	13.5	1, 26	.001

Note. Sex, day, and hour of sampling were entered as fixed factors. Individual identity was entered as random variable ($Z = 3.33$, $P = 0.002$), thus controlling for the repeated sampling effect. No interaction was significant (all $P > 0.10$), therefore being excluded from the final model.

10,000 rpm for 10 min to precipitate the flocculent proteins. The supernatant was examined in a Shimadzu UV-1603 spectrophotometer, and we determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein (Mínguez-Mosquera 1993), the most abundant carotenoid in plasma and commercial food of partridges (J. Garrido, personal communication). Finally, carotenoid concentrations were calculated assuming the described extinction coefficient for this carotenoid pigment in acetone (Mínguez-Mosquera 1993). Plasma samples were analyzed in duplicate, and average values were employed for the analysis.

Analyses were performed by using general linear mixed models (MIXED procedure and REPEATED statement from SAS; Littell et al. 1998). Plasma carotenoid level was introduced as the dependent variable, whereas sex, hour, and day of sampling were entered as fixed factors. The hematocrit variability of samples was included as a covariate in order to account for a possible effect of hemodilution as a consequence of repeated sampling (e.g., Sturkie 1986). Individual identity was included as a random factor. Moreover, in order to analyze hematocrit variability during the experiment, a model including the same factors but testing hematocrit as a dependent variable was performed. Dependent variables met normal distribution and homocedasticity requirements. Tests were two tailed.

Results

Neither the sex nor the hour of sampling during the daylight period (Table 1; Fig. 1, *top*) showed a significant effect on the variability of plasma carotenoid concentration. Meanwhile, the effect of the day of sampling was almost at the limit of significance (two-tailed test, $P = 0.055$; Table 1), with carotenoid levels decreasing throughout the study (Fig. 1, *bottom*). However, such effect could be explained mostly by hemodilution produced by repeated sampling. Thus, hematocrit was positively related to carotenoid variability (estimate parameter ± SE, 0.27 ± 0.07). Moreover, when a backward stepwise procedure was performed, that is, removing nonsignificant terms from the main model, only the hematocrit effect remained ($F = 30.49$, df = 1, 80, $P < 0.0001$). All this was confirmed by the fact that hematocrit variability was also explained by the day of sampling (Table 2), with hematocrit levels decreasing during the study (Fig. 2).

Discussion

Our results suggest that plasma carotenoid levels are not significantly affected by the hour of sampling, at least during daylight period and breeding season, when samples were collected. This is consistent with previous studies in humans that found no (Nierenberg and Stukel 1987) or very small effects (Cantilena et al. 1992) of sampling hour on plasma carotenoid concentration. In contrast, in the only previous study in birds (great tits *Parus major*), Hõrak et al. (2004) found an increase of plasma carotenoid levels with daytime. However, such correlational result was detected at the interindividual level and under field conditions. Therefore, an effect of increased dietary availability of carotenoids as the day progressed could not be excluded. The impact of this potentially confounding factor was reduced here because our captive birds were maintained in a diet with a constant proportion of carotenoids (see "Methods"). Nonetheless, diurnal variation in food intake associated with daily rhythms of activity (also present in wild birds) was not avoided.

However, a significant effect of the day of sampling on plasma carotenoid levels was detected. This effect would be due to hemodilution caused by repeated blood extraction. This is supported by the close relationship between hematocrit and carotenoid values (Table 1) and the similar pattern of hematocrit variability during the study (Fig. 2). In birds, Campbell (1994) recommended a maximum blood sample of 1% of total body mass in order to avoid anemia. The body mass of a red-legged partridge ranges from approximately 400 g for females to 480

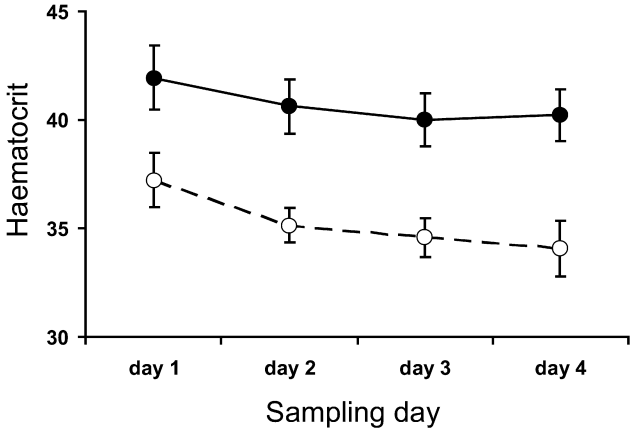

Figure 2. Hematocrit values (%) in relation to day of sampling in male (*filled circles*, *solid line*) and female (*open circles*, *dashed line*) red-legged partridges. Values are means ± SE.

g for males. Here we extracted a total of 2 mL at the end of the experiment (0.5% of body mass), which is far from the proposed limit. Moreover, the fact that hemodilution appeared from the first day of sampling (Fig. 2; first vs. second sample: Scheffé post hoc test, $P < 0.05$) is especially interesting, even when approximately 48 h elapsed between consecutive samplings and when only 0.5 mL had been extracted from each bird. Nevertheless, at the end of the experiment, plasma hematocrit levels decreased only an average of 1.7% and 3.2% in males and females, respectively, which does not seem to carry a serious impact for the health of the individual. However, it should be noted that the decrease in plasma carotenoids was of greater magnitude than that of hematocrit. This may suggest that birds are better able to buffer the effect of blood extraction on red blood cells than on plasma carotenoids or that hemodilution alone cannot totally explain the observed pattern in plasma carotenoids.

Alternatively, the decrease in hematocrit, and consequently, carotenoids, during the experiment could be attributed to an acute and/or a chronic stress response as a consequence of being handled/sampled repeatedly. However, it seems unlikely because each blood sampling took fewer than 2 min per bird in a 48-h period and the cages were visually isolated. Moreover, studies in mammals and fishes suggest that acute stress is in fact related to hemoconcentration and increases (not decreases) in hematocrit values (Allen and Patterson 1995; Bauer et al. 1999; Hoffmayer and Parsons 2001 and references therein). Similarly, experimentally increased levels of corticosterone (the hormone responsible of stress) were also positively related to hematocrit in domestic chickens (Olanrewaju et al. 2006). Finally, to our knowledge, no study has reported any effect of handling stress on hematocrit levels (for a nonsignificant result, see Scope et al. 2002).

To conclude, we must mention that hematocrit showed clear sex-related differences (Fig. 2). Higher hematocrit in males has often been reported in avian bibliography (review in Sturkie 1986), probably being explained by differences in endocrine profiles. Thus, males have higher androgen levels, which are associated with increased erythropoiesis, whereas females have higher estrogen concentrations, which are related to lower erythropoiesis (e.g., Thapliyal et al. 1983; Mooradian et al. 1987). However, in contrast to the findings of Negro et al. (2001), we failed to find significant differences in plasma carotenoids between males and females in this species, which may be attributed to the relatively low number of birds sampled. Alternatively, sexual differences in carotenoid blood levels could be a spurious consequence of higher hematocrit levels of males.

In summary, our results suggest that plasma carotenoid levels do not vary with sampling hour. Therefore, they may be obtained at random during daylight hours with no serious risk of adding variance to data obtained when other sources of variation such as diet, season, age, or sex are controlled for. On the other hand, repeated sampling led to a significant decrease in plasma carotenoids, which should be taken into account in studies involving such kinds of sample collection protocols. Whatever the mechanism involved in the observed decrease (e.g., hemodilution and/or stress), minimizing the volume of the sample collected and establishing a reasonable lapse of time between samplings would be recommendable for experimental designs that require repeated blood extraction. Furthermore, sample volume should be kept constant among individuals and consecutive samplings in order to avoid bias due to differences in hemodilution. Moreover, given the apparent strong relationship between carotenoid blood levels and hematocrit, this last variable should be taken into account in studies measuring the former.

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