

1 **Seasonal variation of red carotenoid pigments in plasma of wild**

2 **Crossbill males *Loxia curvirostra***

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Abstract

Carotenoid-based ornamentation shows great variability in Common Crossbill males. Plumage colour ranges from dull yellow to bright red, but the majority of birds tend to be reddish-orange. It has been suggested that moult in this species is bimodal and that the extent of red feathering depends on the period in which feather development occurred. In this study we analysed the relationship between timing of moult and male plumage colouration in a Common Crossbill population from the Catalanian Pyrenees (NE Spain). Our results demonstrate that the concentrations of dietary carotenoids necessary for the development of red colouration in this species vary significantly within seasons and the main time of feather development is apparently well adapted to the period of highest pigment availability in the environment. The intra-specific colour differences between males might be related to the ability to find food resources rich in red carotenoid precursors and to overcome the physiological processes involved in their metabolism, strongly suggesting that red colouration in Crossbills functions as an honest signal of condition and health status.

Key-words: Common Crossbill, *Loxia curvirostra*, plumage colouration, carotenoids, moult, diet, 3-hydroxy-echinenone.

Introduction

Males of many bird species use carotenoid pigments to colour their feathers red, orange or yellow (Fox 1976). However, they cannot synthesize these compounds *de novo* and must obtain them directly through their diet (Brush 1978; Goodwin 1984). Hence, differences in carotenoid intake at the time of feather development and differences in physiological abilities to accumulate or metabolize ingested pigments may have direct consequences in colour expression (McGraw 2006). Furthermore, carotenoid availability may be significantly influenced by seasonal variation in food quantity and quality, becoming a limited resource at some times of the year (Hill 2006). Consequently, only individual birds that are superior foragers, have a good condition and are able to adapt their annual moult to the abundance of environmental carotenoids will display the brightest colouration (Hill and Montgomerie 1994; Hill 1995; Hill 2006).

Common Crossbills (*Loxia curvirostra*) constitute an ideal model organism to examine the relationship between diet, pigment physiology, timing of feather development and plumage colouration. Adult males display carotenoid-based ornamentation on throat, breast and rump. Colouration varies from dull yellow to bright red, although the majority of birds are reddish-orange (Stradi 1998). The extent of red feathering in male Crossbills appears to be condition-dependent and, as occurs in other cardueline finches, might be used by females to identify high-quality males (Hill 1990; 1991).

Traditionally it has been suggested the timing of moult in Crossbills is bimodal, so that although the main moulting season of yearlings and adults occurs between late summer and the fall, there exist some records of birds starting to moult in early summer as well (Tordoff 1954; Newton 1972; Berthold 1982; Jenni and Winkler 1992). Interestingly, previous descriptive studies had proposed that most males which moulted during the

main moulting period developed red contour feathers, whereas those moulting outside this season tended to grow more yellow or yellowish-green ones (Weber 1972; Ginn and Melville 1983). Common Crossbills are specialist feeders on conifer cones (Nethersole-Thompson 1975) and it has been suggested that these differences in the development of plumage colouration occur at the same time as the birds change from feeding on old cones to new ones, so that perhaps diet might be involved (Newton 1972).

The aims of this study were first to provide quantitative data on the suggested bimodality in the moulting pattern of Common Crossbills and second, to analyse the relationship between timing of moult, plumage colouration and food selection. Blood samples from free-ranging individuals provide a snapshot of recently ingested food carotenoids as well as other pigments that are available for delivery to feathers (Deviche et al. 2008). Hence, circulating plasma carotenoid concentrations of wild male Common Crossbills along the two main moulting periods were used as a proxy of environmental carotenoid availability. Given the suggested relationship between late summer moult and red plumage colouration (Tordoff 1952; Weber 1953) we predicted that circulating levels of the major carotenoids would show maximum levels in late summer.

Material and Methods

Study area

Crossbills were sampled in the Catalanian Pyrenees (1200-2400 m a.s.l) between 1993 and 2006. This area supports abundant resident populations of Crossbills due to the high and steady productivity of Mountain Pine (*Pinus uncinata*) forests, which represent a main food source for this species (Senar et al. 1993; Clouet 2000).

Sample collection

Crossbills were captured with mist nets placed at drinking vessels and when picking up minerals at mountain huts. Birds were individually marked with numbered aluminium rings. Age and sex were recorded according to Svensson (1992) and Jenni & Winkler (1994). Moulting status of every individual was qualitatively determined according to the presence/absence of pinfeathers or growing contour feathers, following the methodology described by Rohwer (1983) and Rohwer & Manning (1990). We scored the general plumage colour pattern of the individuals along a visual scale which ranged from yellow to orange and red (del Val et al. 2009a). We also determined whether the main colour pattern was patched or uniform (del Val et al. 2009a). For analyses on plumage colouration we classified the birds into four visual colour categories: yellow, patched orange, uniform orange and red. The accuracy of this method for colour assessment compared with that of new colourimetric techniques has been demonstrated when distinguishing between these four categories (see del Val et al. 2009a for details): the three apparent colour classes (yellow, orange, red) are mainly related to hue, and to a lesser extent to lightness parameters, whereas the distribution pattern of this colouration (uniform/patched) is mainly associated with chroma.

Carotenoid analysis

Between 2004 and 2006 (March-October) blood samples from 122 yearling and adult males were collected from the brachial vein into heparinized microhematocrit tubes (maximum 400 µl/bird). We kept the samples on coolers and centrifuged them at 11.000 rpm for 10 minutes in the next 8 hours. Plasma was removed, transferred to Eppendorf tubes and frozen at -20°C until analysis by HPLC. Carotenoids were extracted from thawed plasma by adding three parts of acetone (3:1, v:v). The mixtures were introduced in a room temperature bath and sonicated for 5 minutes in order to accelerate the extraction process. We subsequently centrifuged the samples at 13.000 rpm for 10

minutes, obtaining a supernatant with the carotenoids in solution. HPLC was carried out using a Jasco PU-2089 Plus instrument equipped with a quaternary pump (Jasco Analítica Spain, S.L.). Carotenoid analyses were carried out by using a reverse-phase C₁₈ column (Phenomenex Synergi 4μ) and a pre-column of the same material with a particle size of 5 μm. Samples were pre-filtered using OEM filter Nylon (0.45 μm 4mm) and later injected with a Rheodyne 7725i Valve equipped with a 20 μl loop (Rheodyne, Rohnent Park, CA, USA). The eluent system was as the one described in Mínguez-Mosquera and Hornero-Méndez (1993), except that the flow rate was 1 ml min⁻¹. Data were acquired between 195 nm and 650 nm with a multiwavelength detector MD-2010 Plus (Jasco Analítica Spain, S.L.). Yellow carotenoids were quantified using reference lutein obtained from fresh green plants by means of thin layer chromatography (Mínguez-Mosquera 1997) and spectra and retention time of 3-hydroxy-echinenone were compared with those obtained using pure standards. Quantification was performed using external standard calibration curves at 450 nm from injection of progressive concentrations of the reference pigments.

Seasonal changes in the concentration of the main pigments in plasma of Common Crossbill males were examined using ANOVA, with month and moulting stage (moulting/ non-moulting) as main independent factors. We additionally performed planned comparisons to compare circulating levels of these pigments between moulting and non-moulting individuals in specific periods. The relationships between concentrations of different carotenoids were examined using multiple partial correlations, analysing separately moulting and non-moulting males. Analyses showed that the plumage coloration of sub-adult birds differed from that of adults ($X^2_3 = 105.77$, $p < 0.001$, $N = 2212$). Yellow birds were more abundant in the sub-adult class meanwhile uniform-orange and red birds were more abundant in the adult class. We then tested for

an effect of age on the seasonal changes in both lutein and 3-hydroxy-echinenone, but no age effect nor interaction between age and season were detected ($p>0.16$ in all the tests). Because of that, and the fact that sample size did not allow a full design, we removed the age effect from the analysis of seasonal changes in carotenoid content.

Analysis of moult phenology and colouration

The average moult date within the population was determined using 330 males captured along the whole sampling period (1993-2006). We first transformed and expressed the moult date of every bird as the number of days elapsed from the beginning of the moult in the population (earliest date: 1st of March) and the day it was captured moulting. We applied Shapiro-Wilk tests in order to assess whether the frequency distribution of moulting birds departed from a normal distribution.

Given the lack of normality for the dependent variable (timing of moult), the study of the relationship between moult date and plumage colouration was carried out performing a rank transformed ANCOVA (Conover 1981, Conover and Iman 1982), entering age of the birds and annual productivity of Black Pine as covariates. We included the age of the individuals in our analyses because several authors have reported a more frequent occurrence of yellow plumage among yearlings than among older Crossbills, which might be related to differences in the time of moult (Ticehurst 1915; Weber 1953; Newton 1972). The annual productivity of black pines (*Pinus nigra*) was entered as covariate because it may strongly affect the species' phenology in our study area as an extra opportunistic food source (Borràs et al. 2004). Annual productivity of Black Pine forests in our study area was estimated from cone counts in the crowns of sample trees following the methodology described in Borràs et al. (1996). Production was classified as "Low" when no cones were found anywhere (productivity

crash) or some were found in just a few areas and “High” when fructification was locally or generally abundant.

All analyses were performed using the statistical package Statistica 6.0 for Windows and p-level was set at 0.05.

Results

Lutein and 3-hydroxy-echinenone were the main carotenoids contained in Crossbill plasma, whereas zeaxanthin and lutein isomers were less frequently found (Table 1).

We detected no trace of β -cryptoxanthin, the most probable precursor of 3-hydroxy-echinenone, and β -carotene was found in only two adult moulting birds (0.33 and 0.23 $\mu\text{g/ml}$, respectively). We additionally detected a suite of other carotenoids at very low levels that we were unable to identify.

Concentration of lutein varied seasonally in Crossbill males (Table 2; Figure 1a). Moulting individuals showed a significant variation in circulating lutein concentration between months, but this variation was not significant for non-moulting birds (Table 3). Maximum differences in plasma lutein levels between moulting and non-moulting individuals were found in July ($F_{1,92}=10,50$; $p<0.01$; Figure 1a).

Concentration of the metabolically derived red carotenoid 3-hydroxy-echinenone did not vary throughout the year when analysing together birds in any moulting stage (Table 2). However, when moulting and non-moulting individuals were analysed separately we observed a significant seasonal effect on levels of 3-hydroxy-echinenone in moulting Crossbills (Table 3; Figure 1b). Maximum differences in 3-hydroxy-echinenone concentration between these two groups were found in August ($F_{1,84}=11.00$; $p<0.01$).

3-hydroxy-echinenone and lutein were significantly negatively correlated in plasma samples from moulting males. We also found a positive significant link between this red

ketocarotenoid and lutein isomers (Table 4). 3-hydroxy-echinenone concentrations of non-moulting males were positive correlated with levels of lutein, but not with those of other xanthophylls (Table 4).

The first Crossbill males in active body moult within our sample period were found on the 28th of March. The latest birds were observed on the 18th of October. The average moult date within the population was the 19th of August but data did not show a normal distribution (Test Shapiro-Wilk $W=0.93$; $p<0.001$; $N=330$). The frequency distribution of moulting birds throughout the season differed significantly between months ($\chi^2_7=379.61$; $p<0.001$), showing two different moulting peaks, one in June and another more important one in September (Figure 2).

We found a significant effect of plumage colouration on the moulting date of Crossbill males (Table 5). Individuals with uniform-orange and red colouration tended to moult significantly later in the season compared to yellow or patchy-orange birds (Figure 3). The annual productivity of the Black Pine had a significant effect on the moulting dates as well (Table 5). In years with good cone crops males moulted, in average, around the 16th of August (168.09 ± 3.08) whereas in years with low productivity moult occurred around the 21st of July (142.18 ± 4.10). Moulting date depended on the age of the individuals (Table 5), with adult males showing a slight tendency to moult earlier (148.85 ± 9.28 ; $N=184$) than sub-adult birds (175.65 ± 10.00 ; $N=128$).

Discussion

We detected two major carotenoids in the plasma of Common Crossbill males: lutein, a yellow dietary xanthophyll ubiquitous in fruits and green plants (Goodwin 1980) and 3-hydroxy-echinenone, the derived red ketocarotenoid responsible for the red colouration

of their feathers (Stradi et al. 1996; Stradi 1998). As expected, concentration of both pigments showed a significant seasonal variation, especially in moulting individuals. Blood circulation of lutein rose during summer, reaching the highest level in July. This increase was seemingly diet-driven and dependent on the ingestion of carotenoid-rich foods. Previous studies on Common Crossbills in the Pyrenees have reported peaks of seed consumption at the beginning of the summer (i.e., July). The species exploits unripe pine cones at this time of the year, extracting the milky seeds from the unopened cones (Gènard and Lescourret 1987; Clouet 2000). They also consume other seasonal food sources, such as buds (Gènard and Lescourret 1987) and pine shoots (Newton 1972; Pulliainen 1974). These green plant parts are especially rich in yellow xanthophylls (del Val et al. 2009c) and might therefore be responsible of the summer increase in circulating lutein levels observed in our Crossbill population. Maximum 3-hydroxy-echinenone concentration was found in August, coinciding with the main peak of moult of the species. This seasonal variation may be the result of the specific production of this pigment at the time of feather development. This may explain why it mostly appeared in moulting birds. Moreover, the fact that the highest levels of 3-hydroxy-echinenone appear one month later in relation to the maximum availability of dietary carotenoids might indicate production and accumulation of red ketocarotenoids in the liver when the precursors are abundant in the environment and subsequent mobilization at the time of moult (del Val et al. 2009b, c). 3-hydroxy-echinenone is the main carotenoid responsible for red colouration in Crossbill males (Stradi 1998). This pigment is converted from dietary yellow precursors in the liver, transported via bloodstream to feather follicles and deposited into growing feathers during moult (del Val et al. 2009b). Based on parsimonious chemical modifications, previous studies have proposed β -cryptoxanthin as the most likely non-

xanthophyll precursor of this red ketocarotenoid (Stradi et al. 1996). In a preceding work on White-winged Crossbills (*Loxia leucoptera*) these authors discussed whether this precursor may be directly acquired through the diet or may constitute a metabolically formed product of other dietary carotenoids in this species (e.g. lutein, Deviche et al. 2008). In a recent study on the carotenoid content in Crossbills diet, we analysed by HPLC extracts of several potential food sources exploited by the species in our study area, including pine cones, buds, shoots, strobili, pollen and various fruits (see del Val et al. 2009c for details). β -cryptoxanthin was present in small amounts in the majority of the samples, and therefore we might assume that Crossbills acquire this limited precursor through the diet and metabolize it into 3-hydroxy-echinenone during moult to become colourful (del Val et al. 2009c). However, no trace of β -cryptoxanthin was found in our blood samples.

The absence of β -cryptoxanthin in Crossbill plasma might be related to its scarcity in diet. Besides their role as integumentary colorants, carotenoids have important functions in many physiological processes. They can work as antioxidants, immunomodulators and photoprotectants, or take part in vitamin synthesis and intercellular communication (McGraw 2006). Consequently, when a scarce colorant such as β -cryptoxanthin becomes available in Crossbills blood transport system, a conflict between physiological functions and ornamentation is entirely possible. Due to the important role of β -cryptoxanthin in feather pigmentation, the species might have developed the ability of absorbing this molecule rapidly as soon as it is available and deliver it to the liver for converting it into 3-hydroxy-echinenone and deposited in feathers (del Val et al. 2009b, c). Despite the absence β -cryptoxanthin in circulation, we may assume that all ingested pigments of dietary origin increase with the amount of carotenoid-enriched food resources consumed and hence, show the same seasonal variation observed for

lutein. For this reason, we used the pattern obtained for lutein as a reference to explain the availability of potential dietary precursors (e.g. β -cryptoxanthin) all over the year. We cannot exclude completely the possibility that β -cryptoxanthin might constitute a metabolically formed product of lutein, as previously advanced for zebra finches (*Taeniopygia guttata*) (McGraw et al. 2002). Interestingly, we found a strong negative correlation between lutein and 3-hydroxy-echinenone in the plasma of moulting Crossbills, which might indicate a potential precursor-product relationship between these two pigments (McGraw et al. 2002). The fact that the correlation did not appear in non-moulting birds additionally stresses this point. The absence of β -cryptoxanthin in plasma might then be explained assuming a local hepatic transformation of this molecule into 3-hydroxy-echinenone. The retinoid-carotenoid links could also explain the negative relationship between 3-hydroxy-echinenone and lutein, since β -cryptoxanthin and 3-hydroxy-echinenone are pro-vitamin A but lutein is not (Hill and Johnson 2012). However, further work would be required in future to resolve these physiological questions.

In accordance with previous descriptive literature (Berthold and Gwinner 1972; Newton 1972; Rimkevich 1990; Jenni and Winkler 1994), moult followed a clear bimodal pattern in our Common Crossbill population. We found two main moulting peaks during the year, one in early summer (June) and another one in September, involving the main fraction of the population. As expected, most males with yellow or patchy-orange colouration tended to moult earlier in the season compared to those with redder plumage and higher percentage of 3-hydroxy-echinenone in feathers (Weber 1972; Ginn and Melville 1983). Apparently, the aforementioned seasonal availability of certain dietary components is involved in the observed variability in contour feather colour (Newton 1972; Jenni and Winkler 1994). From winter to late spring Crossbills from our

study area feed basically on open cones of black and mountain pines (Clouet 2000). Pine seeds have a high energetic value (Gènard and Lescourret 1987) but are poor in non-xanthophyll carotenoids (Goodwin 1980; McGraw et al. 2001). Therefore, birds that moult early in the season would lack potential precursors necessary to develop a bright red colouration. In early summer, mountain pines from subalpine/alpine areas start to flower and birds may exploit a great variety of new resources, such as buds, shoots, pollen or unripe seeds that they extract from unopened cones. The caloric value of these food sources is relatively low (Gènard and Lescourret 1987) but contain significant amounts of lutein and β -cryptoxanthin (del Val et al. 2009c). Consequently, Crossbills moulting in the main period would have potentially higher availability of non-xanthophyll precursors and might incorporate higher proportion of red carotenoids to colour their plumage.

Our findings indicate that Common Crossbill males tend to adjust the time of feather development to the period of maximum carotenoid availability in the environment in order to maximize colour production and become more sexually attractive to females (Hill 1995). However, this adjustment to environmental conditions would contribute only in part to explain the intra-specific variation in the expression of plumage colouration in this bird species (Hill 2000). The development of red colouration on Crossbills entail more metabolic costs in comparison to yellow and orange pigmentation due to the biochemical expenditure involved in the oxidation of non-xanthophyll precursors (Hill 1996). As mentioned above, the energetic content of green plant parts is relatively low in comparison with that of ripe pine seeds (Gènard and Lescourret 1987) hence, birds moulting in late summer might be confronted with a clear trade-off: the higher availability of carotenoid precursors *versus* the low calorific value of the sources. Only birds in best condition could face the energetic constraints of these physiological

processes in the second moulting period and subsequently display a healthy and colourful red plumage (Hill 2000).

We have focused along the paper on the view that pigment access explains the patterns we have observed, and this is not necessarily the only explanation for results found. Feather colour might be a signal of overall condition, not just pigment access (Hill 2011), and mistiming of moult may be another indication of poor system homeostasis that links to colour through condition. Hence, birds might be drab because they are poor at metabolizing yellow dietary pigments, not because they have insufficient yellow pigments. However, we think that the presence of a bimodal distribution of moult, with one peak related to the availability of high energy resources and the other to the presence of dietary and metabolized carotenoids, strongly suggest a trade-off probably related to carotenoid and general resources availability. If intra-specific colour differences between males were related to the ability to find food resources rich in red carotenoid precursors, this could allow these skilful birds to overcome the physiological processes involved in their metabolism. If this was the case, it would strongly suggest that red colouration in Crossbills functions as an honest signal of condition and health status.

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Tables

Table 1 Content ($\mu\text{g/ml}$) of different carotenoids in the plasma of Crossbill males (N=122, except for 3-hydroxy-echinenone: N=114) in the Catalanian Pyrenees.

	Mean	SE	Minimum	Maximum
Lutein	1.44	0.20	0.0	9.39
3-hydroxy-echinenone	0.53	0.21	0.0	20.28
Zeaxanthin	0.10	0.02	0.0	1.33
Lutein isomers	0.31	0.06	0.0	3.27
Non-identified	0.16	0.07	0.0	6.40

Table 2 Summary of a two-way ANOVA analysis showing the effect of moulting stage (moulting/non-moulting) and month on circulating levels of lutein and 3-hydroxy-echinenone in plasma of Crossbill males in the Catalanian Pyrenees.

	df	F	p
Lutein			
Moulting stage	1, 92	3.02	0.09
Month	4, 92	6.91	< 0.001
Moulting stage x Month	4, 92	2.02	0.10
3-hydroxy-echinenone			
Moulting stage	1, 84	0.81	0.37
Month	4, 84	1.99	0.10
Moulting stage x Month	4, 84	2.71	0.04

Table 3 Planned comparisons on the variation of circulating lutein and 3-hydroxy-echinenone in Crossbills from the Catalanian Pyrenees, according to their moulting stage (moulting/ non-moulting).

	df	F	p
Moulting			
Lutein	4, 51	8.10	<0.001
3-hydroxy-echinenone	4, 50	3.05	0.03
Non-moulting			
Lutein	6, 59	1.38	0.24
3-hydroxy-echinenone	6, 54	0.98	0.45

Table 4 Partial correlations between 3-hydroxy-echinenone and other plasma carotenoids in Crossbill males from the Catalanian Pyrenees.

	Partial corr.	t(51)	p
Moulting			
Lutein	-0.58	-5.11	<0.001
Lutein isomers	0.62	5.61	<0.001
Zeaxanthin	0.27	2.02	0.05
Non-moulting			
Lutein	0.28	2.18	0.03
Lutein isomers	-0.03	-0.22	0.82
Zeaxanthin	-0.07	-0.51	0.61

Table 5 Results of a rank transformed ANCOVA testing the effect of plumage colouration (yellow, patchy-orange, uniform-orange, red), age (adult, yearling) and annual productivity of Black Pine (good crop, bad crop) on the moulting date of Crossbill males in the Catalanian Pyrenees.

	df	F	p
Colour	3, 296	4.47	<0.01
Black Pine fructification	1, 296	19.24	<0.001
Age	1, 296	3.86	0.05
Age x Colour	3, 296	2.43	0.07
Black Pine fructification x Colour	3, 296	1.83	0.14
Black Pine fructification x Age	1, 296	0.03	0.86
Black Pine fructification x Age x Colour	3, 296	0.66	0.57

Figure legends

Fig. 1 Seasonal changes in lutein and 3-hydroxy-echinenone plasma concentrations (means \pm SE) of Common Crossbill males from the Catalanian Pyrenees.

Fig. 2 Variation in the percentage of moulting Crossbill males throughout the year in the Catalanian Pyrenees ($\chi^2_{7}= 379.61$, $p < 0.001$).

Fig. 3 Average moult date for different colour categories of Crossbill males in the Catalanian Pyrenees (media \pm SE). Asterisks refer to significance based on planned comparisons between the different colour categories ($p < 0.05$). No differences in the average moult date were found when comparing uniform orange and red individuals (n.s.).

Figure 1

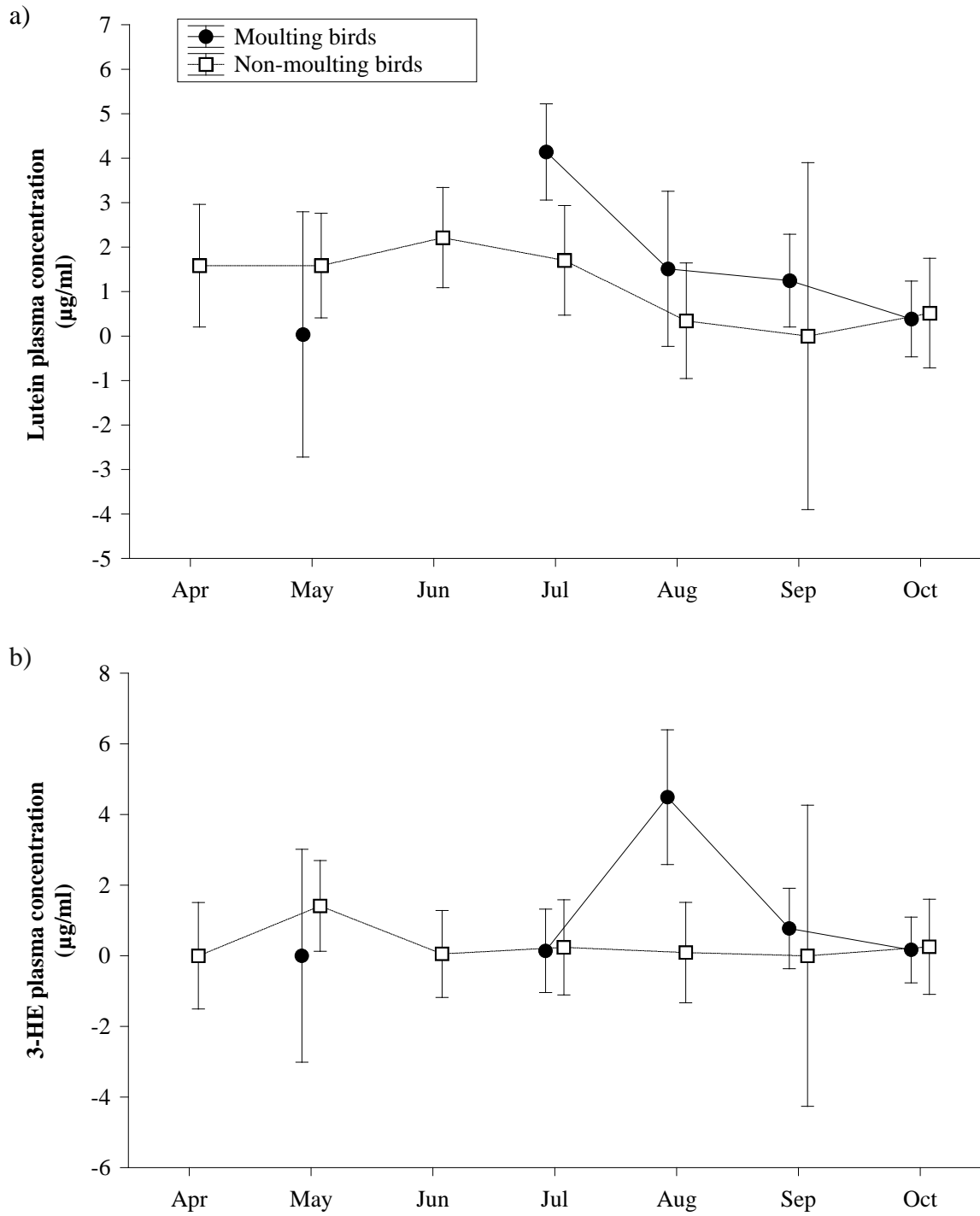


Figure 2

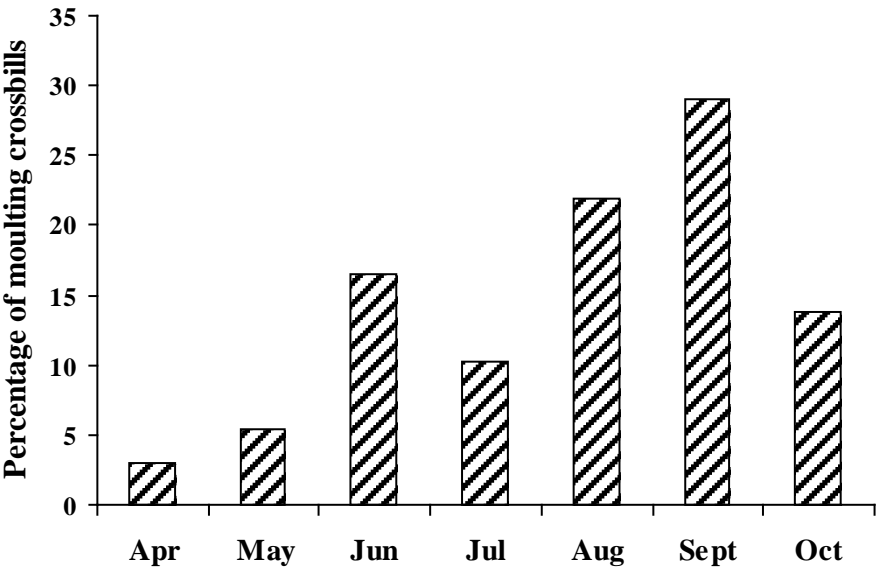


Figure 3

