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Beyond topology: coevolution of structure and flux in metabolic networks

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Abstract

Interactions between the structure of a metabolic network and its functional properties underlie its evolutionary diversification, but the mechanism by which such interactions arise remains elusive. Particularly unclear is whether metabolic fluxes that determine the concentrations of compounds produced by a metabolic network, are causally linked to a network's structure or emerge independently of it. A direct empirical study of populations where both structural and functional properties vary among individuals' metabolic networks is required to establish whether changes in structure affect the distribution of metabolic flux. In a population of house finches (*Haemorrhous mexicanus*), we reconstructed full carotenoid metabolic networks for 442 individuals and uncovered 11 structural variants of this network with different compounds and reactions. We examined the consequences of this structural diversity for the concentrations of plumage-bound carotenoids produced by flux in these networks. We found that concentrations of metabolically derived, but not dietary carotenoids, depended on network structure. Flux was partitioned similarly among compounds in individuals of the same network structure: within each network, compound concentrations were closely correlated. The highest among-individual variation in flux occurred in networks with the strongest among-compound correlations, suggesting that changes in the magnitude, but not the distribution of flux, underlie individual differences in compound concentrations on a static network structure. These findings indicate that the distribution of flux in carotenoid metabolism closely follows network structure. Thus, evolutionary diversification and local adaptations in carotenoid metabolism may depend more on the gain or loss of enzymatic reactions than on changes in flux within a network structure.

Introduction

Evolutionary diversification of metabolic networks has been attributed to both structural changes in the occurrence of enzymatic reactions and compounds as well as functional changes in the concentrations of compounds produced by the same network structure (Jeong *et al.*, 2000; Almaas *et al.*, 2005; Badyaev *et al.*, 2015; Nidelet

et al., 2016), but the mechanistic links between these structural and functional properties remain elusive (Stelling *et al.*, 2002; Papp *et al.*, 2009; Lee *et al.*, 2012a). The tempo and mode of evolutionary changes in metabolism depend on the correspondence between the structure of a metabolic network and the relative rates of each of the reactions in the network, known as metabolic flux, that determine the concentrations of compounds in the network (Torres-Sosa *et al.*, 2012); the strength of these interactions determines whether changes in compound concentrations can occur under constant network structure, or only when reactions or compounds are gained or lost. Thus, examination of the correspondence between the distribution of flux among compounds and structural changes in the network that produces these compounds

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can uncover the mechanisms that underlie local adaptations in metabolism.

When flux coevolves with the structural positions of enzymatic reactions (Wright & Rausher, 2010; Rausher, 2013), only changes in metabolic network structure would lead to variation in the rates of reactions (Emmerling *et al.*, 2002; Lee *et al.*, 2012b; Piedrafita *et al.*, 2015). For example, the flux of a substrate compound can be partitioned differently among the same reactions when there are changes in the number of additional reactions associated with the substrate (Fig. 1a; Fell & Thomas, 1995; Rossell *et al.*, 2006; Nilsson & Nielsen, 2016). Under this scenario, the structure of a metabolic network determines functional properties of metabolism and thus evolves for a specific distribution of flux among compounds (Fig. 1a; Wagner, 2003; Eloundou-Mbebi *et al.*, 2016). Adaptive changes in the relative concentrations of compounds produced by metabolic networks would therefore be dependent on the evolution of different network structures, caused by variation in the occurrence of substrate compounds that cannot be metabolically derived (Borenstein *et al.*, 2008; Kreimer *et al.*, 2008), or the evolution of novel enzymatic reactions and loss of existing reactions (Wagner, 2012).

Alternatively, the function of a metabolic network could be decoupled from its structure (Hartl *et al.*, 1985;

Lee *et al.*, 2012a; Inoue & Kaneko, 2013), and reaction rates can change without gain or loss of enzymatic reactions or substrate compounds (Kacser & Acerenza, 1993; Eanes, 1999; Almaas *et al.*, 2004; Flowers *et al.*, 2007; Olson-Manning *et al.*, 2013). This scenario would produce a different distribution of flux among the same compounds within a constant network structure, depending on which reactions rates change within a network (Fig. 1b; Shimizu *et al.*, 2003; Shirai *et al.*, 2005). In this case, we would predict that changes in network structure would not be related to changes in the distribution of flux among compounds (Fig. 1b). If the distribution of flux is decoupled from metabolic network structure, then adaptive changes in the production of compounds would be dependent on the regulation of existing enzymatic reactions (reviewed in Morrison & Badyaev, 2016a).

Comparing the structure of metabolic networks and compound concentrations among individuals from the same population allows for the direct examination of the proximate effects of the gain or loss of enzymatic reactions on the distribution of flux among compounds while controlling for confounding effects of substrate availability and other factors (Kim & Ryu, 1999; Sauer *et al.*, 1999; Iyer *et al.*, 2008). Here, we studied the metabolic network that produces plumage carotenoids in birds to test whether

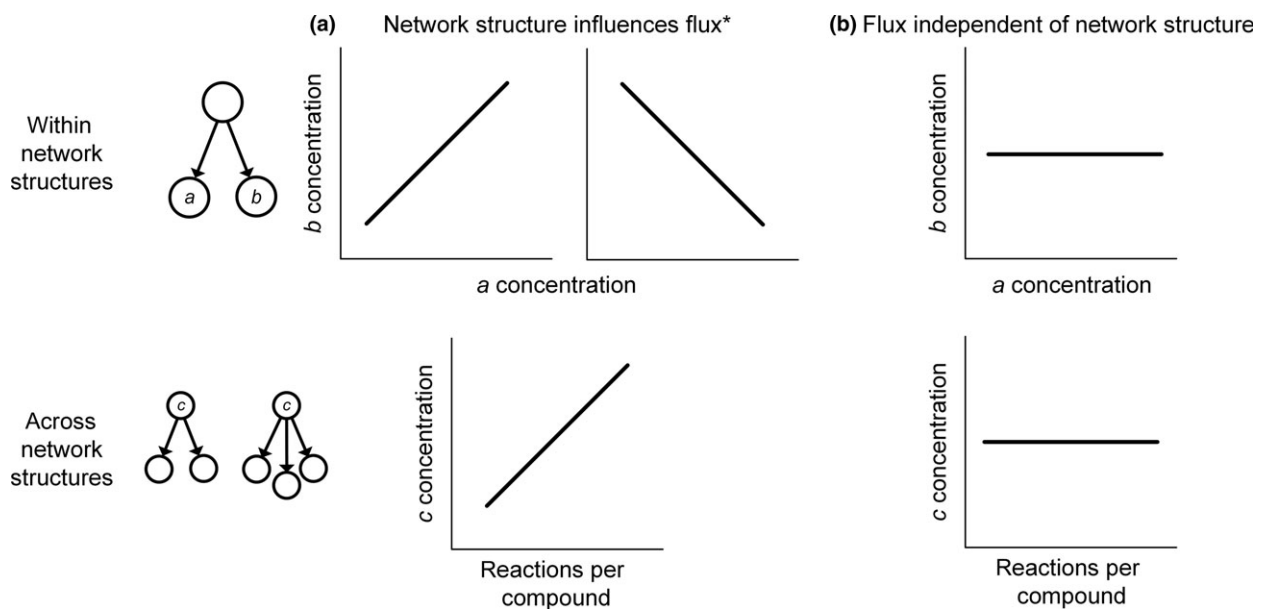


Fig. 1 The correspondence between network structure and flux underlies metabolic diversification. Network structure is defined by the number of reactions (arrows) connecting compounds (circles). The concentrations of compounds represent functional properties of flux. (a) If the structure of a network corresponds to a specific distribution of flux, then there should be proportional changes between the concentrations of compounds in the same network structure (top panel). Changes in the number of reactions per compound would proportionally redistribute flux among compounds, and compound concentrations would correspond to distinct network structures (bottom panel). (b) If flux can be partitioned in different ways among the same compounds in a network structure, then changes in the concentrations of compounds would not be correlated within a network structure (top panel). As a result, flux would be partitioned similarly among compounds in different network structures, leading to the lack of correspondence between the concentration of a compound and the number of reactions (bottom panel). The asterisk denotes prediction supported by this study.

the variation in carotenoid concentrations is caused by the gain or loss of reactions, or by changes in the regulation of the same enzymes in a constant metabolic network structure.

We studied the association between network structure and metabolic flux of plumage-bound carotenoids in a population of house finches (*Haemorrhous mexicanus*). First, we built metabolic networks underlying the production of plumage carotenoids for each individual and quantified metabolic flux for 12 plumage carotenoids. Second, in order to determine the relationship between network structure and function in carotenoid metabolism, we examined whether the concentration of each compound varied on a local scale with number of reactions directly connected to the compound, or on a global scale in distinct network structures comprised of different groups of directly and indirectly connected compounds and reactions. Changes in the concentration of compounds in response to structural changes would indicate that functional properties correspond to specific network structures (Fig. 1a), while the absence of a relationship between compound concentration and network structure would mean that the distribution of flux among compounds is independent of network structure (Fig. 1b).

We then tested whether the relative strength of the relationship between network structure and flux for a compound depended on its structural position in the metabolic network. Birds have to obtain the initial substrates for carotenoid metabolism from their diet (Brush, 1981), and if the dietary carotenoids are present in individuals' diets in excessive amounts (e.g. Koch *et al.*, 2016), then their concentrations should be less associated with changes in network structure than that of metabolically derived carotenoids. Additionally, compounds with the highest enzymatic connectivity are involved in the production of most derived compounds and their loss can disrupt a network's function (Albert *et al.*, 2000; Jeong *et al.*, 2001), such that the flux of these compounds could be under selection for robustness to changes in network structure (Kim *et al.*, 2007). If this is the case, we would expect the concentrations of compounds with the greatest enzymatic connectivity to be less dependent on network structural changes than compounds with the fewest reactions.

We further examined variation in compound concentration among individuals with the same network structure. Strong correlations among compound concentrations within a network structure indicate that flux is partitioned similarly among compounds, while weaker correlations indicate that fluxes of individual compounds are independent of structure. If weaker correlations among compounds occur in networks with high among-individual differences in carotenoid concentration, then variation in flux partitioning occurs on static network structures. Alternatively, if networks

with the strongest correlations between compounds have the most variable carotenoid concentrations, then variation in carotenoid concentrations among individuals with the same network structure is associated with global, proportional changes in the production of compounds across individuals with the same metabolic network. Based on our findings, we discuss the contribution of structural and functional properties of carotenoid metabolism to intraspecific variation in compound concentrations and consider the implications of these results for the evolutionary diversification of metabolic networks.

Materials and methods

Study population

We analysed 1326 feather samples from 442 adult male house finches (*H. mexicanus*) in an individually colour-marked study population in southeastern Arizona from 2003 to 2013 (protocol of feather processing and fieldwork in Landeen & Badyaev, 2012). For each male, we sampled three to five feathers from each ornamental area (breast, rump and crown) and processed the feathers from each ornament separately. Methods for feather carotenoid extraction, analysis, identification and quantification are in Higginson *et al.* (2016).

House finch carotenoid metabolic network

House finches' plumage contain up to 19 expressed carotenoids, including seven dietary carotenoids (β -carotene, β -cryptoxanthin, α -carotene, gazaniaxanthin, lutein, rubixanthin, zeaxanthin) and 12 carotenoids derived by the metabolism of dietary carotenoids (α -doradexanthin, β -isocryptoxanthin, 3'-dehydrolutein, 3'-hydroxy-echinenone, 4-oxo-rubixanthin, canary xanthophyll A, canary xanthophyll B, adonirubin, adonixanthin, astaxanthin, canthaxanthin, echinenone) (Inouye *et al.*, 2001; McGraw *et al.*, 2006; Higginson *et al.*, 2016). When all observed and intermediate compounds and reactions known for the species are considered together, they form a metabolic network consisting of 24 compounds and 45 enzymatic reactions (Fig. 2a; Badyaev *et al.*, 2015; Morrison & Badyaev, 2016b). In this study, we focused on a subset of 12 dietary and derived carotenoids that are linked by enzymatic reactions: β -carotene, β -cryptoxanthin, lutein, zeaxanthin, β -isocryptoxanthin, 3'-dehydrolutein, 3'-hydroxy-echinenone, adonirubin, adonixanthin, astaxanthin, canthaxanthin and echinenone.

Construction of individual metabolic networks

For each individual, we selected the plumage sample that contained the maximum number of carotenoid compounds. When all samples of an individual had the

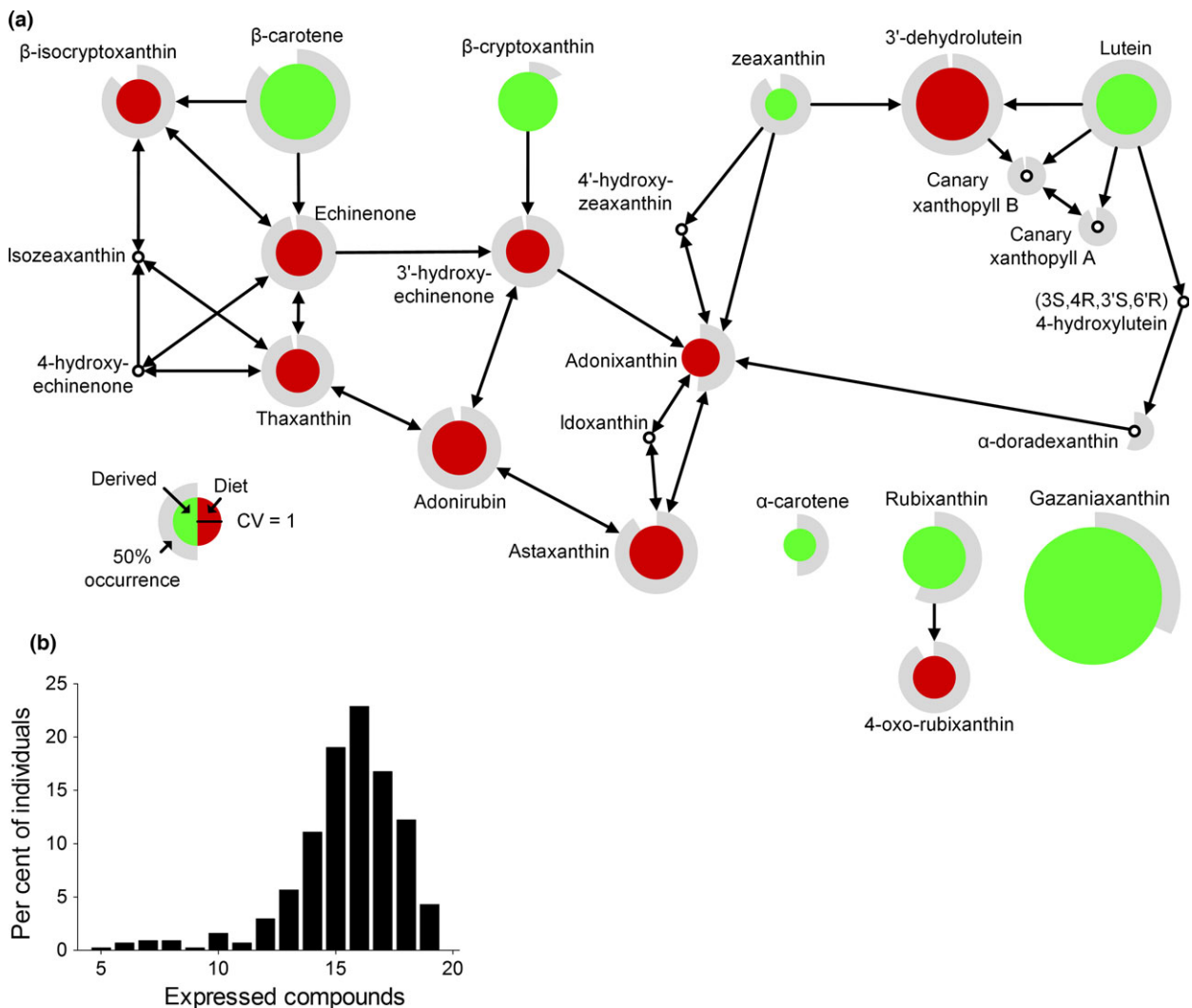


Fig. 2 Occurrence and concentration of plumage carotenoids in male house finches. (a) The species' carotenoid metabolic network includes 24 compounds (circles) and 45 reactions (arrows). Grey shading indicates the percentage of $n = 442$ individuals in which the carotenoid occurs, and the diameter of the circles indicates the among-individual coefficients of variation (CV) of compound concentration ($\mu\text{g/g}$ pigmented feather). Dietary carotenoids (light green circles) are the start of pathways that produce metabolically derived carotenoids (dark red circles). Small open circles show carotenoids not examined in this study. (b) The number of compounds identified in each individual.

same number of compounds, a sample was selected at random. To construct an individual metabolic network, we mapped carotenoids identified in an individual sample on the full enzymatic network for the species (see Badyaev *et al.*, 2015 and Morrison & Badyaev, 2016b, for justification). The mapping of groups of observed carotenoids resulted in 48 distinct network structures.

Network structural measures

For each network structure, we calculated the numbers of incoming and outgoing reactions, the pathway

position, enzymatic connectivity and betweenness centrality (C_b) for each compound. The pathway position of a compound was the average of the minimum number of sequential reactions the compound is from each of the dietary carotenoids (β -carotene, β -cryptoxanthin, lutein, zeaxanthin) in the species' network. Dietary carotenoids were assigned a pathway position of zero reactions. The enzymatic connectivity of a compound was the total number of incoming and outgoing reactions directly associated with a compound in the species' network and is a local measure of compound connectivity. Betweenness centrality (C_B) of a compound (n) – a

global structural measure of connectivity was defined as

$$C_B(n) = \sum_{s \neq n \neq t} (\sigma_{st}(n) / \sigma_{st}),$$

where s and t are compounds different from n , σ_{st} represents the number of shortest pathways from s to t , and $\sigma_{st}(n)$ is the number of shortest pathways from s to t that include n (Brandes, 2001). Betweenness centrality describes how often a compound (n) falls in the shortest path between pairs of compounds and thus represents the influence of a compound on other network compounds (Yoon *et al.*, 2006). We calculated the betweenness centrality using Cytoscape 2.8.2 (Smoot *et al.*, 2011) with NetworkAnalyzer 2.7 (Assenov *et al.*, 2008; Doncheva *et al.*, 2012).

Statistical analyses

We log-transformed concentrations to achieve normal distribution. General linear models were used to test for differences in compound concentrations among individuals that vary in incoming or outgoing reactions and for differences in the concentrations of a compound among network structures. Pairwise differences between least-squares mean concentrations with different incoming or outgoing reactions, and between compound concentrations in unique network structures were tested using the Tukey–Kramer procedure (Kramer, 1956) due to unequal numbers of individuals per group. The standardized pairwise mean difference, also known as the effect size, between the concentrations of a compound associated with different numbers of incoming or outgoing reactions and between the concentrations of a compound in unique network structures, was measured using Cohen's d (Cohen, 1962), defined as the difference between two group means (\bar{x}_1 and \bar{x}_2) divided by the pooled standard deviation of the two groups:

$$d_{12} = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1+n_2-2}}},$$

where s^2 is the variance and n is the number of samples in a group. We then ranked the effect sizes of structural changes on the concentrations of each compound and tested whether the magnitude of the change in the concentration of a compound in response to the addition or loss of reactions was correlated with a compound's pathway position, connectivity and betweenness centrality.

For each network structure, we constructed linear principal components (PC) based on a correlational matrix of concentrations of individual carotenoids. We examined the eigenstructure of these matrices to compare the patterns of correlation among individual carotenoids within and across each network structure.

All statistical analyses were carried out using SAS v. 9.4.

Results

Within-species variation in network structure and metabolic flux

Occurrence of the 19 carotenoids expressed across 442 individuals varied from 14.03% (dietary β -cryptoxanthin) to 100% (dietary lutein) (Fig. 2a), and individuals expressed from 6 to 19 carotenoids in their feathers (Fig. 2b). Of the subset of 12 dietary and derived carotenoids that formed a continuous network, 48 distinct network structures (distinct combinations of dietary and derived compounds) occurred at least once. All but one of the 12 carotenoids (91.67%) varied in connectivity (number incoming or outgoing reactions) across these network structures (Fig. 3). Among individuals, the concentration of dietary zeaxanthin varied the least (coefficient of variation, CV = 0.662; Fig. 2a) and the concentration of dietary ganaxanthin varied the most (CV = 2.84; Fig. 2a).

Local changes in network structure redistributed flux among compounds

The response of flux, as measured by compound concentrations, to local changes in network structure, as measured by the number of reactions directly connected to a compound, varied among compounds (Table S1). Concentrations of dietary carotenoids did not change with the number of outgoing reactions (Fig. 3a–c; lutein: $F_{2,439} = 0.55$, $P = 0.577$; zeaxanthin: $F_{1,412} = 1.32$, $P = 0.251$; β -carotene: $F_{1,382} = 1.87$, $P = 0.173$). The concentrations of five (62.50%) of the derived carotenoids varied with the number of reactions connected to a compound, but each type of structural change in reactions did not have the same effect for all compounds. The concentrations of two of the three compounds with paired changes in incoming and outgoing reactions depended on the number of reactions connected to the compounds (Fig. 3d–f; echinenone: $F_{1,422} = 23.04$, $P < 0.001$; adonirubin: $F_{1,423} = 8.68$, $P = 0.003$; 3'-hydroxy-echinenone: $F_{1,432} = 0$, $P = 0.967$). Similarly, the concentrations of two of the three compounds associated with changes in the number of outgoing reactions varied with the number of reactions (Fig. 3f,j–k; 3'-hydroxy-echinenone: $F_{1,432} = 56.11$, $P < 0.001$; astaxanthin: $F_{1,383} = 0.94$, $P = 0.334$; canthaxanthin: $F_{1,421} = 31.66$, $P < 0.001$). The concentration of only one of the four compounds with different numbers of incoming reactions was affected by the number of reactions (Fig. 3f–i; 3'-hydroxy-echinenone: $F_{1,432} = 1.34$, $P = 0.247$; 3'dehydrolutein: $F_{1,438} = 3.35$, $P = 0.068$; β -isocryptoxanthin: $F_{1,389} = 3.48$, $P = 0.063$;

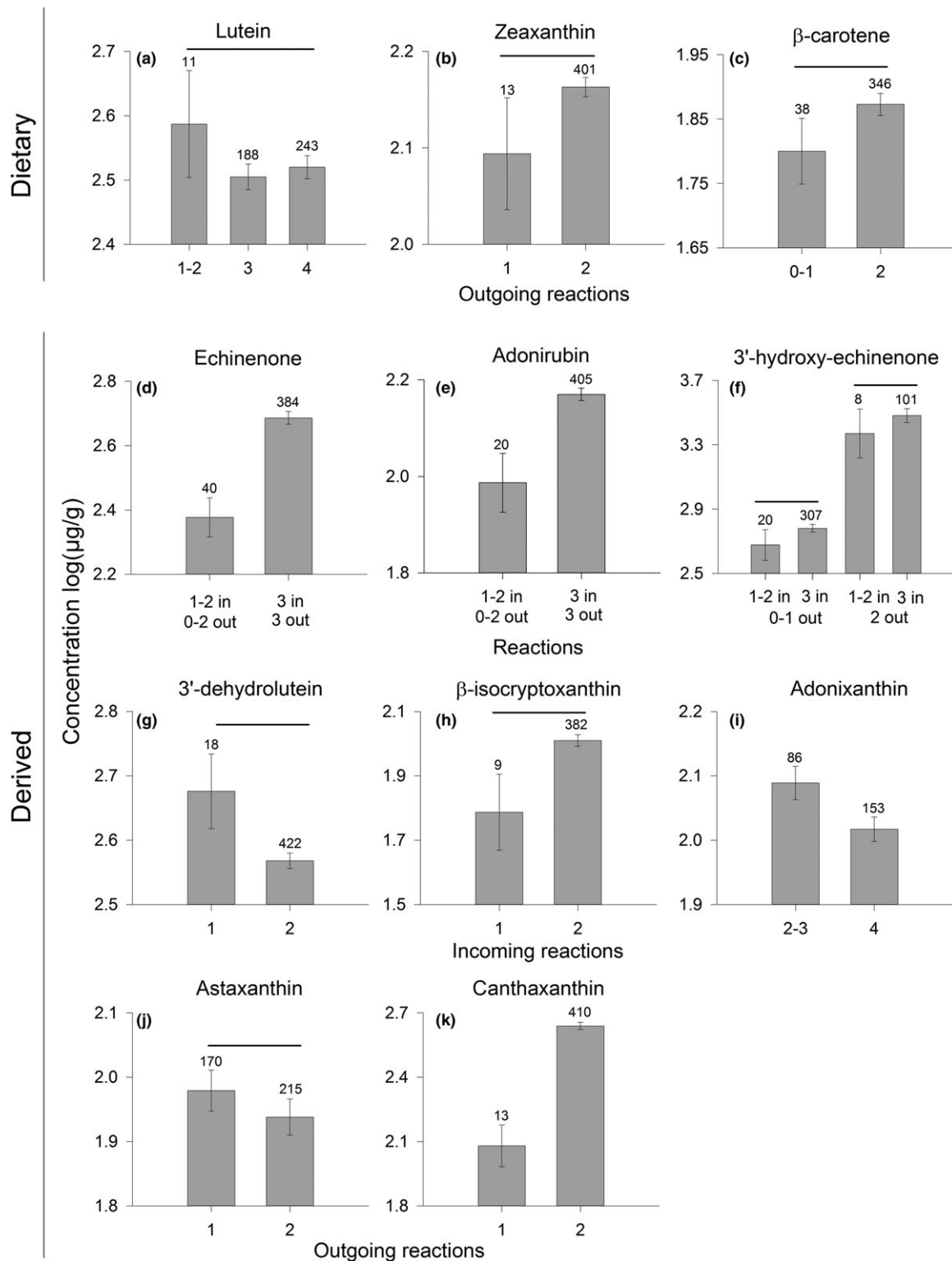


Fig. 3 Metabolic flux of compounds is associated with their local structural properties. Shown are the least-squares means (± 1 standard error, SE) for the concentrations of dietary (a–c) and derived (d–k) carotenoids partitioned by number of incoming (in) or outgoing (out) reactions per compound. Horizontal lines indicate no difference between groups at $P < 0.05$. The numbers above each bar are sample sizes. See Table S1 for a summary of the statistics.

adonixanthin: $F_{1,236} = 5.00$, $P = 0.026$). Overall, the concentrations of derived compounds varied more than that of dietary compounds when these compounds were associated with different numbers of reactions (Fig. 4a; Spearman $\rho = 0.602$, $P = 0.05$, $n = 11$). Meanwhile, changes in compound concentration associated with network structural changes were not correlated with local (Fig. 4b; $\rho = 0.463$, $P = 0.15$) or global (Fig. 4c; $\rho = 0.200$, $P = 0.55$) measures of compound connectivity.

Global changes in network structure redistributed flux among compounds

Of the 48 distinct network structures that occurred at least once across the 442 individuals, 11 occurred in at least five individuals and were used in further analyses of global network structure. Concentrations of six of the 12 carotenoids differed among network structures (Fig. 5). Two of these were dietary (β -carotene: $F_{7,337} = 2.51$, $P = 0.016$ and lutein: $F_{10,374} = 3.60$, $P < 0.01$); and four were derived (β -isocryptoxanthin: $F_{8,361} = 5.39$, $P < 0.001$; canthaxanthin: $F_{10,374} = 3.62$, $P < 0.001$; echinenone: $F_{10,374} = 3.56$, $P = 0.0002$ and 3'-hydroxy-echinenone: $F_{10,374} = 3.02$, $P = 0.001$). Of the six carotenoids whose concentrations did not vary among network structures, two were dietary (β -cryptoxanthin: $F_{2,51} = 0.06$, $P = 0.94$ and zeaxanthin: $F_{9,370} = 0.92$, $P = 0.51$) and four were derived (3'-dehydrolutein: $F_{10,374} = 1.36$, $P = 0.195$; adonirubin: $F_{10,374} = 1.01$, $P = 0.432$; adonixanthin: $F_{5,219} = 1.09$, $P = 0.367$; and astaxanthin: $F_{8,355} = 1.51$, $P = 0.154$). The total concentration of all of the compounds expressed by individuals also varied with network structure ($F_{10,374} = 2.44$, $P = 0.008$). The lowest concentrations of most of the compounds occurred in

the absence of dietary zeaxanthin (network D) (Fig. 5 and Table S2). The highest concentrations of compounds occurred in network structures without derived carotenoids (networks E, G, J) (Fig. 5 and Table S2).

Distribution of metabolic flux was constant within a network structure

The distribution of metabolic flux among compounds was conserved across individuals within each of the 11 networks; the concentrations of all compounds were strongly correlated (Fig. 6 and Table S3). Furthermore, across-network differences in the relative strength of the correlations between the same compounds in the first two principal components (PC1 and PC2) of each network structure demonstrated that the distribution of flux varies across structures (Fig. 6 and Table S3). Within each network structure, PC1 reflected positive correlations between all of the compound concentrations, suggesting that most of the among-individual variation within a network is the result of proportional and unidirectional variation in compound concentrations (Fig. 6 and Table S3). The negative correlations between several of the compounds reflected by PC2 in each network structure mean that some among-individual variation within a network structure was caused by trade-offs in the production of compounds, such that the increase in concentration of one compound is associated with the decrease in concentration of another compound (Fig. 6 and Table S3). The largest among-individual differences in total carotenoid concentration occurred in network structures with the strongest correlations and most conserved distributions of flux among compounds (Fig. 7; $t = 2.29$, $b_{ST} = 0.607$, $P = 0.048$, $n = 11$).

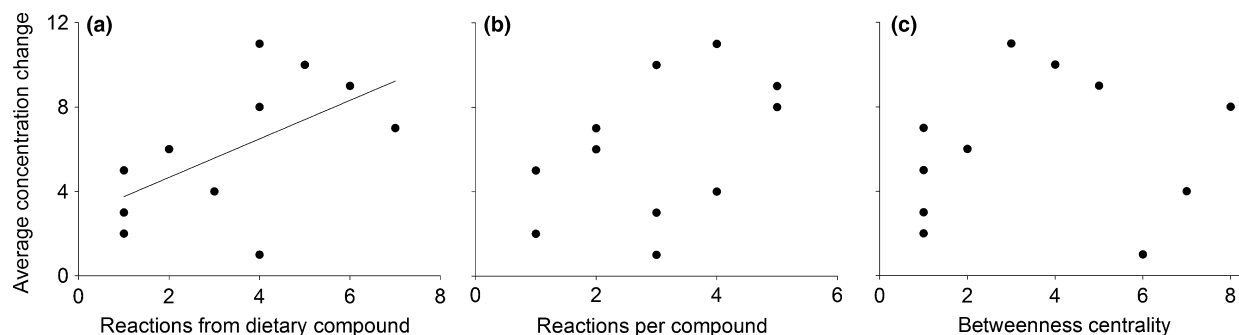


Fig. 4 The effect of network structure on flux of a compound depends on a compound's pathway position, but is not associated with its local or global connectivity. (a) The number of reactions affected concentrations of derived compounds more than dietary compounds. Differences in the connectivity of compounds in the species network, as measured locally by (b) number of reactions and globally by (c) betweenness centrality, were not related to the effect of changes in the number of reactions on compound concentration. The number of reactions from a dietary compound separates starting dietary compounds (0 reactions) from derived compounds (> 0 reactions from dietary compounds). The average concentration change is the absolute value of the mean difference (Cohen's d) between concentrations of compounds with different numbers of reactions.

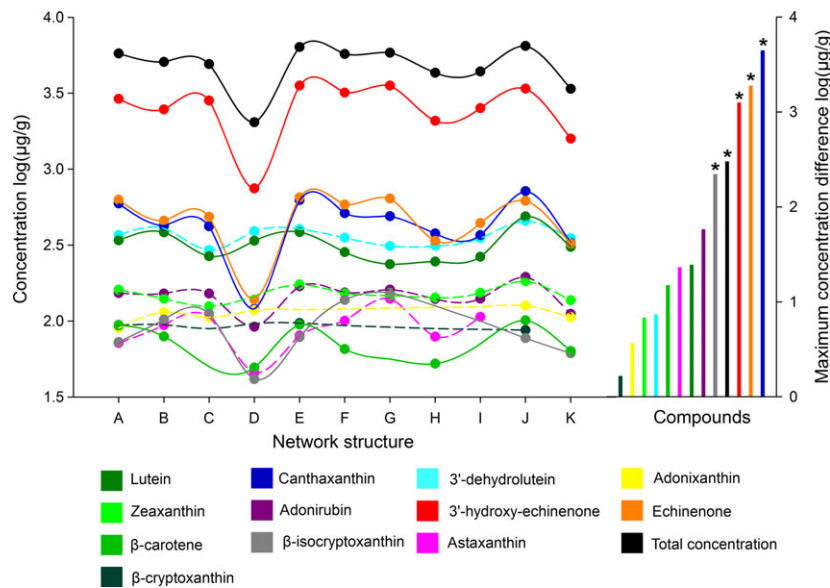


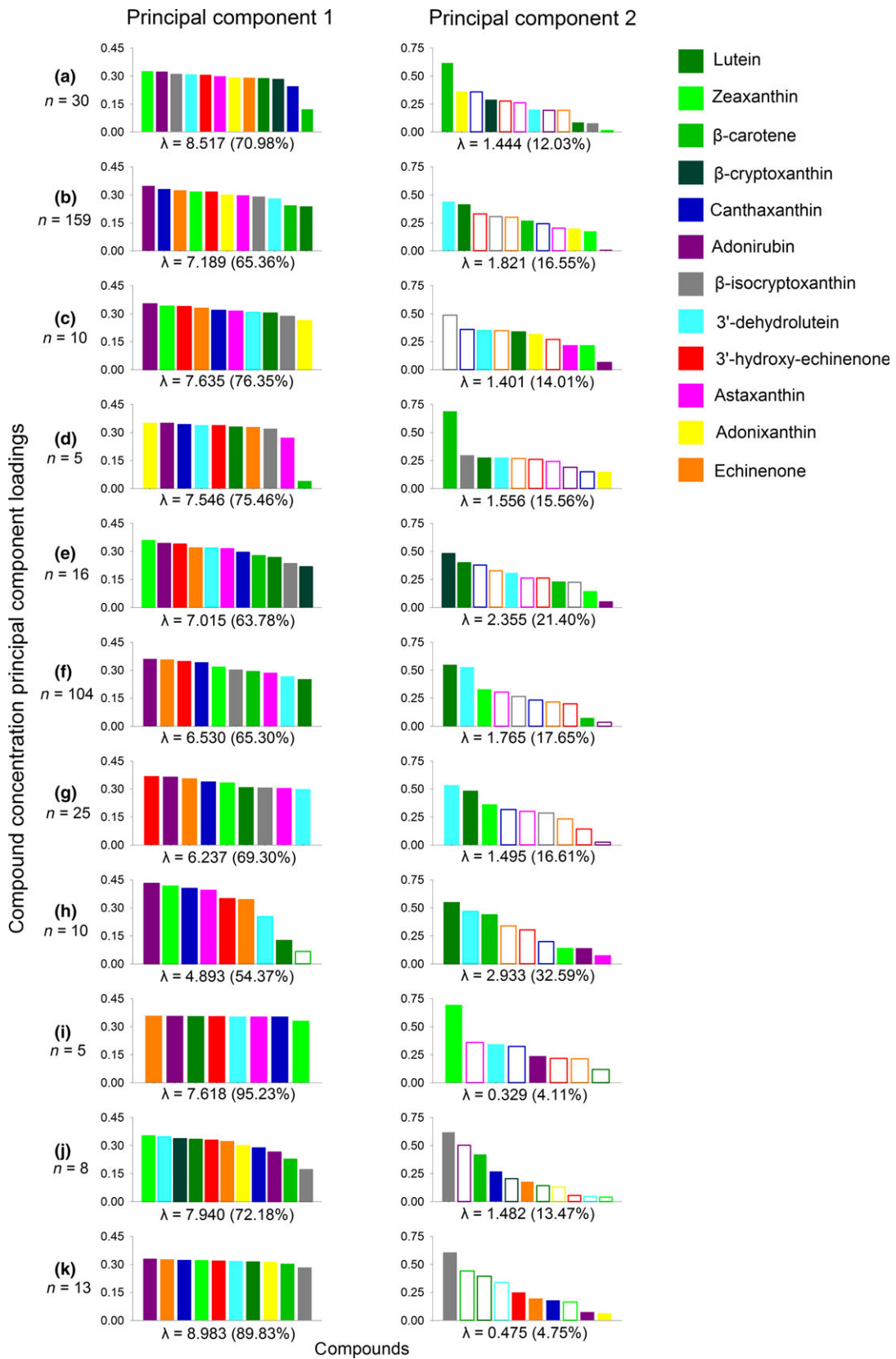
Fig. 5 Metabolic flux is distributed differently among network structures. Shown are the least-squares means for the concentrations of compounds across network structures (left). Solid lines show compounds whose concentrations differed among network structures and dashed lines show compounds whose concentrations did not vary across network structures. Dietary carotenoids are shown in shades of green, the total concentration (sum of all compound concentrations in the network) in black, and the remaining colours represent derived carotenoids. Network structures ($n = 11$) were as follows: A = all compounds present; B = β -cryptoxanthin absent; C = β -cryptoxanthin and β -carotene absent; D = β -cryptoxanthin and zeaxanthin absent; E = adonixanthin absent; F = adonixanthin and β -cryptoxanthin absent; G = adonixanthin, β -cryptoxanthin and β -carotene absent; H = adonixanthin, β -isocryptoxanthin and β -cryptoxanthin absent; I = adonixanthin, β -isocryptoxanthin, β -cryptoxanthin and β -carotene absent; J = astaxanthin absent; K = astaxanthin and β -cryptoxanthin absent. Shown on the right is the maximum difference (Cohen's d effect size) between the least-squares means of the concentration of a compound in different networks (Table S3). The asterisk denotes significant pairwise differences in compound concentration between networks at $P < 0.05$. See Table S2 for a summary of the statistics.

Discussion

To what extent do structural properties of a metabolic network reflect its functional properties? We found that the structure of a metabolic network influences how carotenoid flux is partitioned among compounds: concentrations of derived compounds varied with reaction number (Fig. 3d–f,i,k; Table S1) and among distinct network structures (Fig. 5). The association between flux and network structure arose because flux was partitioned similarly among compounds within each network structure (Fig. 6 and Table S3). Differences in the strength of correlations between compound concentrations across network structures further demonstrated that the distribution of flux among the compounds follows network structure

(Fig. 6 and Table S3). The largest variation in compound concentrations among individuals occurred in network structures that had the strongest correlations of flux among compounds (Fig. 7), suggesting that differences in carotenoid concentrations within the same network are caused by the proportional change in the production of all of the compounds in the network at the same time, and not by isolated changes in the flux of only a few compounds. Taken together, these findings support the prediction that the gain or loss of enzymatic reactions affects concentrations of expressed carotenoids more than changes in the distribution of flux among compounds on the same network structure and suggest that the structural and functional properties of carotenoid metabolism have coevolved (Fig. 1a).

Fig. 6 Metabolic flux is partitioned similarly among compounds in all individuals with the same network structure. Concentrations of compounds in a metabolic network were closely correlated, but the strength of correlations differed among network structures. Panels show correlations of each compound concentration in PC1 (left column) and PC 2 (right column) for each network structure (a–k) (see the legend of Fig. 4 for network descriptions). The eigenvalue for each PC and the per cent of variation explained by it (in parentheses) are below each panel. Dietary compounds are shown in shades of green and the remaining colours represent derived compounds. Filled and unfilled bars denote positive and negative correlations, respectively. The number below each panel letter shows the number of individuals with this network structure. See Table S3 for a summary of the statistics.



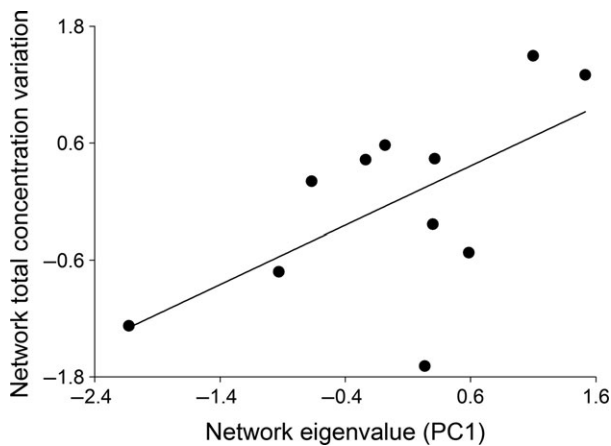


Fig. 7 The greatest among-individual variation in total carotenoid concentrations occurs in network structures with the most conserved distribution of flux among compounds. In individuals with the same structure, compound concentrations varied the most in networks with the strongest correlations between compounds. This establishes that reactions in these structures are regulated as a single module and the concentrations of all of the compounds in the network proportionally change together. Concentration variation is the standard deviation of the total carotenoid concentrations (sum of all of the compound concentrations in a network) among individuals with the same network structure.

How does the gain or loss of reactions and compounds influence flux partitioning, and when should flux be decoupled from the structure of a network? We found that changes in network structure redistributed flux among compounds. These findings corroborate results of studies showing shifts in flux with activation or inactivation of reactions [e.g. in glycolysis, Ralser *et al.* (2007), or carbon flux, Shi *et al.* (2010)]. In our study, flux dependency on global and local network structural properties differed among derived compounds, and only some changes in network structure corresponded to changes in compound concentration (Figs 3 and 5). For example, flux was not equally repartitioned among the remaining compounds in the network following the loss of either dietary or derived compounds (Fig. 5; Table S2). Differences in the effect of structural changes on compound concentration could depend on the relative rates of flux of the other reactions in the network or on the amount of substrates available for further metabolism. If the enzyme activity of an additional reaction is coregulated by the same mechanisms as other reactions associated with a compound (Kacser & Burns, 1973; Mazat *et al.*, 1996), then the same amount of flux should be partitioned among more compounds, due to the conservation of mass in enzymatic reactions. Thus, when the amounts of substrates remain the same, the concentration of a compound should decrease with the addition of outgoing reactions (Brooks, 2004), as we observed in adonixanthin (Fig. 3i). On the contrary, the concentrations of

the other derived compounds increased with the number of reactions (Fig. 3d–f,k). This could be caused by an increase in the flux to these compounds, achieved by higher reaction rates of incoming reactions from other derived compounds (Kacser & Acerenza, 1993; Yang & Robb, 1994; Wehtje & Adlercreutz, 1997), or consuming a greater amount of a dietary compound (Wu *et al.*, 2006; Taymaz-Nikerel *et al.*, 2011, 2013). The latter mechanism was supported by our finding that the total concentration of carotenoids (representing the mass available for carotenoid metabolism from the diet), differed between network structures (Fig. 5; Table S2). Alternatively, the addition of reactions could inhibit the activity of other reactions directly associated with a compound (Kacser & Burns, 1981). This would cause a decrease in the production of the compounds associated with these reactions, and so the amount of the substrate partitioned into products would remain the same.

We found that flux of derived carotenoids depended on network structure more than flux of dietary carotenoids (Fig. 4a), suggesting that excess of externally acquired dietary compounds could mask the effects of the gain or loss of reactions on concentrations of their products. Indeed, birds consume more dietary carotenoids than they deposit into feathers or use to produce derived carotenoids (Fox *et al.*, 1969; Koch *et al.*, 2016). The independence of dietary compounds and network structure likely accounts for some of the divergence of correlational structures between concentrations of dietary and derived carotenoids among networks (Fig. 6; Table S3).

The absence of dietary compounds, however, directly affected the concentrations of many of the derived carotenoids (Fig. 5 and Table S2). For example, the loss of the dietary zeaxanthin was associated with the lowest concentrations of almost all of the derived compounds across all network structures. In the house finch carotenoid network, derived carotenoids are located fewer reactions away from zeaxanthin than from other dietary compounds (Fig. 2a). If shorter pathways of sequential reactions are energetically cheaper, then the production of derived compounds from zeaxanthin may be the most energy efficient strategy for the metabolism of derived compounds in comparison with the three other dietary compounds in house finches (Britton, 1976; Brush, 1981). The importance of zeaxanthin in maintaining avian carotenoid metabolism is further supported by the finding that some species preferentially accumulate higher proportions of zeaxanthin than other dietary compounds (McGraw *et al.*, 2004). In this study, we found that zeaxanthin was the most prevalent and the least variable of the dietary carotenoids (Fig. 2a), suggesting stabilizing selection for both the structural and functional contributions of dietary zeaxanthin to carotenoid metabolism in this population.

Strength of the association between network structure and metabolic flux can reflect the functional necessity of some compounds in different environments. Environmentally induced structural changes, such as fluctuations in the quantity of externally acquired compounds, can result in the redistribution of flux among products in the network (Handorf *et al.*, 2005; Borenstein *et al.*, 2008). Some compounds must always need to be produced despite environmental fluctuations (Barkai & Leibler, 1997; Batchelor & Goulian, 2003; Kim *et al.*, 2007; Shinar *et al.*, 2009), whereas others are associated with specific environments (diCenzo *et al.*, 2016). The avian carotenoid network structure includes redundant metabolic pathways from dietary to derived compounds (Badyaev *et al.*, 2015) that facilitates the robustness of some compounds to changes in dietary inputs (Ma *et al.*, 2009; Shinar & Feinberg, 2010; Eloundou-Mbebi *et al.*, 2016; Gao *et al.*, 2016). We expected compounds connected to the production of the greatest number of derived compounds to be more robust to network structure than compounds associated with the production of only a few derived compounds. However, we found that neither local nor global measures of compound connectivity accounted for the dependency between a compound's structural properties and flux (Fig. 4b,c). This corroborates empirical findings that robustness of compound concentrations to environmental changes is conferred by the global interactions between all of the compounds in a network, and not by the structure of directly associated reactions (Ma *et al.*, 2004; Inoue & Kaneko, 2013).

The finding that network structure accounted for variation in concentrations of plumage carotenoids at the population level suggests that the gain or loss of enzymatic reactions and dietary compounds is crucial for the evolution of local adaptations involving plumage-bound carotenoids. The observed variation in the occurrence and concentration of carotenoids among individuals from the same population (Fig. 2a) suggests that activation and deactivation of enzymatic reactions can be accomplished rapidly and is easily modulated (see also Badyaev & Duckworth, 2003). Reversible regulatory changes could be driven by the environment during moult (Szasz, 1974; Ralser *et al.*, 2007; Link *et al.*, 2013), or by hormonal inhibition or activation (Cohen, 1988; Strålfors & Honnor, 1989), and these allow for short-term structural changes and rapid adaptation of carotenoid metabolism. Within recurrent environments, these regulatory changes in enzyme activity may become permanent (Emilsson *et al.*, 2008; Gordon & Ruvinsky, 2012; Schaeffe *et al.*, 2013; Lopes *et al.*, 2016; Mundy *et al.*, 2016), leading to the evolution of distinct structures of metabolic networks.

The coevolution of network structure and properties of flux delineates possible trajectories of evolutionary diversification of carotenoid metabolism. Within all networks under this study, individuals differed the most when compound concentrations changed proportionally

(Fig. 6; Table S3). This suggests that groups of interconnected compounds form functional modules in which flux is globally regulated by the same mechanism across all of the reactions in the module (Fell & Thomas, 1995; Rossell *et al.*, 2006). Indeed, the greatest among-individual variation in flux occurred in network structures that had the strongest positive correlations among compound concentrations, which represent the most conserved distributions of flux among compounds (Fig. 7). The modular regulation of interconnected compounds suggests that selection acts on the regulation all of the enzymes in a pathway. This is reflected in the evolutionary diversification of avian carotenoid metabolism, where the gain and loss of entire biochemical modules was more common than incremental gains or losses of individual compounds (Morrison & Badyaev, 2016b).

The mechanisms underlying changes in the structure of a biochemical network could also determine how and where diversification occurs in the network. In house finch carotenoid metabolism, only a small proportion of flux variation among individuals with the same network was caused by trade-offs among compound concentrations (Fig. 6; Table S3), and so the loss of a compound due to the increased production of another does not seem to be a source of structural changes in this population. Novel enzymatic reactions are commonly added to compounds that already directly or indirectly produce derived compounds in the network (Barabási & Albert, 1999; Jeong *et al.*, 2000; Barabási & Oltvai, 2004; Light *et al.*, 2005), and this could constrain most of the evolutionary changes in flux partitioning to only the most connected compounds in the network. Thus, we would expect periodic bursts of diversification in flux partitioning in network locations with the fewest enzymatic reactions.

This study is one of the first comprehensive assessments of the correspondence between network structure and flux in a multicellular organism. Importantly, we found substantial variation in network structure and flux among individuals within the same population, likely over similar genetic architecture underlying the biochemical network. We found that changes in network structure likely play a more definitive role in local adaptation and diversification of carotenoid metabolism than functional properties of compound concentration. The range of documented carotenoid network structures might allow for rapid adaptations of metabolic flux to changes in structural properties of the network, either as a result of environmental change or fitness consequences of the resulting products, ultimately facilitating further coevolution of structural and functional network properties.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Results of the pairwise Tukey–Kramer tests between the compound concentrations of individuals partitioned by different numbers of reactions per compound.

Table S2 The maximum change (Cohen’s *d* effect size) in the concentration of a compound, log(μg compound/g feather), between distinct network structures.

Table S3 Results from the principal component analyses of compound concentration variation among all of the compounds present in a unique network structure.

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