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Emergent buffering balances evolvability and robustness in the evolution of phenotypic flexibility

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Evolution of adaptive phenotypic flexibility requires a system that can dynamically restore and update a functional phenotype in response to environmental change. The architecture of such a system evolves under the conflicting demands of versatility and robustness, and resolution of these demands should be particularly evident in organisms that require external inputs for reiterative trait production within a generation, such as in metabolic networks that underlie yearly acquisition of diet-dependent coloration in birds. Here, we show that a key structural feature of carotenoid networks–redundancy of biochemical pathways–enables these networks to translate variable environmental inputs into consistent phenotypic outcomes. We closely followed life-long changes in structure and utilization of metabolic networks in a large cohort of free-living birds and found that greater individual experience with dietary change between molts leads to wider occupancy of the metabolic network and progressive accumulation of redundant pathways in a functionally active network. This generated a regime of emergent buffering whereby greater dietary experience was mechanistically linked to greater robustness of resulting traits and an increasing ability to retain and implement previous adaptive solutions. Thus, experience-related buffering links evolvability and robustness in carotenoid-metabolizing networks and we argue that this mechanistic principle facilitates the evolution of phenotypic flexibility.

KEY WORDS: **Age, degeneracy, evolvability, network, phenotypic flexibility, robustness.**

PHENOTYPIC FLEXIBILITY AND THE TRANSITIONS BETWEEN ROBUSTNESS AND EVOLVABILITY

For evolution to proceed, the maintenance of a currently adaptive configuration of traits must not preclude innovation in the same traits (Baldwin 1902; Schmalhausen 1938; Lewontin 1970; West-Eberhard 2003). The conceptual resolution of this problem envisions an evolutionary lineage traversing a continuous landscape of linked robust networks, each delineating a functional phenotypic state (Maynard Smith 1970). To the extent that alternative phenotypic states are reachable from the previous state and survivable (Bershtein et al. 2006; Draghi et al. 2010; Lässig et al. 2017), they are retained in an uninterrupted evolutionary lineage. Since the introduction of this visionary principle by the founders of evolutionary theory (e.g., Schmalhausen 1938; Dobzhansky 1940), it has been empirically and conceptually corroborated and enriched by numerous studies (reviewed in Maynard Smith 1970; Gavrilets 2004; Wagner 2011; Wagner 2014; Peter and Davidson 2015). Although empirical studies have clearly established the centrality of this principle to explaining biological patterns at vastly different levels of organization (Huynen et al. 1996; Fontana and Schuster 1998; Weinreich et al. 2006; Povolotskaya and Kondrashov 2010; Sarkisyan et al. 2016), we know very little about the specific processes and mechanisms behind these patterns, especially at microevolutionary timescales. For example, how do switches between phenotypic states occur in development? Or how are previously successful functional solutions preserved and incorporated when the current environment favors them (Chetverikov 1926; Stebbins and Hartl 1988; Müller and Wagner 1991)? Answers to these questions require a direct study of phenotype reorganization and the associated transition between robust

phenotypic states to observe how preexisting functional configurations can be integrated with new environmental inputs to form a continuous lineage of phenotypes.

The study of phenotypic flexibility—environmentally influenced phenotypic adjustment ("updating") that occurs repeatedly during the lifetime of an individual (Piersma and Drent 2003; West-Eberhard 2003)—provides such an opportunity, because it allows direct insight into integration of an existing phenotype, new environmental inputs, and inherited components in modification of adaptive features (Bateson et al. 2014; Stamps and Krishnan 2014; Nettle and Bateson 2015; Beaman et al. 2016; Panchanathan and Frankenhuis 2016; Stamps and Frankenhuis 2016). Indeed, recent studies of age-dependent phenotypic adjustments that integrated life-history, developmental, genetic, and informational perspectives have provided some of the most illuminating insights into both the mechanistic basis of within-generation modifications (e.g., Fagiolini et al. 2009; Remedios et al. 2017) and optimal phenotypic adjustment in relation to current costs and benefits (Kokko 1997; Cichoñ 2001; Frankenhuis and Panchanathan 2011; Fischer et al. 2014; English et al. 2016; Stamps and Krishnan 2017).

In this study, we describe a process that reconciles the robustness needed for functioning of a phenotype, with its flexibility during reiterative reorganization of the phenotype over a lifetime. We specifically focus on the role of metabolic redundancy—one of the most common mechanisms behind phenotypic robustness (Wagner 2005; Papp et al. 2009). We capitalize on three aspects of our study system—the known structure of a metabolic network that underlies phenotype, environment-dependency of trait production that necessitates differential utilization of the network in each iteration of phenotype production, and life-long monitoring of phenotypic outcomes in relation to network utilization, environmental change, and reorganization of prior phenotypic states.

BIOCHEMICAL PATHWAY REDUNDANCY IN PRODUCTION OF ENVIRONMENTALLY DEPENDENT TRAITS

Functioning of any phenotype includes metabolism of precursors that cannot be internally synthesized, but must be obtained from the environment. When functioning in a different environment requires a different combination of biochemical pathways that metabolize these environmental precursors, the enzymatic network readily evolves modularity (Milo et al. 2002; Kashtan and Alon 2005), where each module consists of a set of interconnected pathways starting with externally acquired (dietary) metabolites and ending with a derived product needed for organismal functions (e.g., Borenstein et al. 2008; Kreimer et al. 2008). An organism's (or a trait's) persistence across environments that vary in dietary precursors is facilitated by biochemical pathway redundancy (also called pathway degeneracy) in which an identical metabolite is interchangeably produced from different precursors (e.g., Edelman and Gally 2001; Tanaka et al. 2006; Vitkup et al. 2006). Redundant compounds are then produced when any one of their dietary precursors is encountered, and switches between the pathway modules are achieved by nonspecific enzymes (Srere 1987; Khosla and Harbury 2001; Morrison and Badyaev 2016a). Such network organization arises as a compromise between environment-specific function of each biochemical module and the transition between modules when the environment changes—making it a model system in which to study a balance between robustness and evolvability of a phenotype that is produced by such a network.

LIFE-LONG UPDATING OF CAROTENOID PATHWAYS DURING ANNUAL MOLTS IN BIRDS

A prime example of such a balance is carotenoid-producing networks in birds. Birds cannot convert noncarotenoids to carotenoids (Goodwin 1984), but instead obtain external, environment-specific, carotenoids with diet during each molt, metabolize them to variable degrees and deposit them into plumage. Avian carotenoid networks vary widely in size and complexity (McGraw 2006; Badyaev et al. 2015), but are necessarily comprised of biochemical modules, each starting with a dietary carotenoid and linked to downstream carotenoids though a series of enzymatic reactions (Morrison and Badyaev 2016b). Over evolutionary time, frequent gains and losses of dietary precursors lead to gains and losses of entire corresponding biochemical modules (Badyaev et al. 2015; Morrison and Badyaev 2016b), such that evolutionary diversification of avian carotenoid networks is dominated by recombination of conserved biochemical modules, with patterns of enzymatic connectivity within each module remaining largely intact (Toews et al. 2017). Biochemically redundant carotenoids, located at the intersections of two or more pathway modules, play a key role in this process. By acting as bridges between the diet-specific modules, they sustain ongoing metabolic elongations of pathways despite fluctuations in dietary carotenoids (Badyaev et al. 2015, 2017), allow access to a vast connected network of available metabolic phenotypes and thus underlie spectacular diversification in carotenoid-based plumage (Morrison and Badyaev 2016b), and account for long-term coevolution of carotenoid metabolism with other organismal traits (Higginson et al. 2016).

The same principle should apply on an ecological time frame: redundancy of biochemical pathways should convey robustness to carotenoid ornamentation by interchangeably using pathways depending on available dietary precursors and, therefore, absorbing environmental fluctuations in dietary inputs across molts or environments. Further, theory suggests that biochemical redundancy should facilitate retention and incorporation of previous functional associations into the active network (Edelman and

Gally 2001; Wagner 2008; Shinar and Feinberg 2010; Barve and Wagner 2013). A test of these ideas requires the direct study of the interaction between structure and utilization of metabolic networks in relation to variable environmental inputs and phenotypic outcomes.

PREDICTIONS FOR THE ROLE OF BIOCHEMICAL PATHWAY REDUNDANCY IN PHENOTYPIC FLEXIBILITY

We assume a scenario under which individuals utilize a subset of diet-specific biochemical modules when exposed to a particular set of dietary carotenoids during each molt (Fig. 1), which is supported by a recent empirical study (Morrison and Badyaev 2017). Increasing experience with a diversity of dietary inputs across molts (hereafter—dietary change) is necessarily reflected in greater occupancy of the individual's metabolic network over its' lifetime, but this experience can only be translated into greater potential for plasticity when past solutions are retained and can be incorporated into development. This is accomplished when prior experience with a particular dietary carotenoid affects its metabolism in subsequent molts of life (such as by increasing efficiency of an enzyme involved in the conversion). In the case of biochemically redundant pathways, the use of a subset of enzymatic pathways primes the entire production module (Fig. 1).

We investigated the occurrence and consequences of such priming by testing three scenarios for phenotypic flexibility in diet-dependent carotenoid ornamentation (Fig. 1). Under the "no updating" scenario, the phenotypic flexibility reflects variable dietary inputs utilized by an invariant network structure, under the "complete updating" scenario, phenotypic flexibility reflects a variable network structure that itself follows dietary inputs, and under the "partial updating" scenario, flexibility is shaped by both current and preceding changes in network structure and dietary inputs. Under all three scenarios, biochemical pathway redundancy can compensate for variation in either inputs or the network structure and produce consistent ornamentation (Fig. 1), however direct examination of the structure and utilization of the metabolic network (Fig. 2) enables us to test the mechanisms behind the three predictions. Further, the focus on within-individual replicates of trait development under the framework of Fig. 1 allows us to distinguish between age-related and experience-related phenotypic flexibility.

We tested these predictions by studying life-long changes in successive ornamentation and its underlying metabolic origins in a large cohort of free-living, individually marked house finches (*Haemorhous mexicanus*). We first show that flexible use of the metabolic network absorbs fluctuations in dietary inputs between successive molts and this accounts for consistent and predictable life-long trends despite fundamental diet-dependency of carotenoid-based ornamentation. Second, we show how biochemical pathway redundancy allows previous experience with dietary change to be retained in metabolic networks, leading, in turn, to a greater ability to capitalize on previous adaptive solutions, and greater robustness of resulting traits later in life. We argue that such experience-related buffering facilitates evolution of phenotypic flexibility and discuss the remarkable parallelism between the roles of redundancy in reconciling versatility and robustness on vastly different evolutionary scales.

Materials and Methods

DATA COLLECTION

We followed life-long changes in successive ornamentation in a cohort of 74 free-living house finches (general field protocols in Badyaev and Vleck 2007). The males within this cohort were first marked as local juveniles before their first molt and then measured every year from their first molt of life (postjuvenile molt) to their fourth molt. We limited our data to four consecutive molts because it accurately represents a natural lifespan for the house finches that survive their first winter (Badyaev et al. 2012) and because we needed at least two episodes of trait development for intermediate ages to assess the dependencies between them (see below). Each of the 74 males was captured after it completed each molt and 10–15 ornamental feathers (3–5 from each of the three ornamental areas—crown, breast, and rump) were collected.

Males were photographed on a standardized photostand with a high resolution camera against a neutral gray background with scale markings and a color scale (protocol, measurement error, and repeatability analyses for the method are in Badyaev and Duckworth 2003). Ornamented patch area, hue, and saturation were obtained from the resulting files with SigmaScan Pro 5.0 (SPSS, Inc.). Briefly, for hue and saturation, a 10×10 pixel grid was overlaid on each image and one pixel was sampled in each square of the grid that covered the ornamented patch using the hue function of SigmaScan. This technique allowed us to sample most feathers of the ornament patch and to obtain an accurate representation of the overall hue and saturation (averaged across an ornament). We inverted and rotated the color wheel such that males with highest score for hue were most ornamented (i.e., no color $= 0^{o}$).

Seven dietary and 13 derived carotenoids (Fig. S1) were extracted and identified in the feather samples following protocols in Higginson et al. (2016). When considered together, these carotenoid compounds and their metabolic precursors form a species-specific network of 25 nodes connected by 45 reactions [Fig. S1, specific enzymes for each reaction are in Appendix S2 of Badyaev et al. (2015)]. Individual samples contained between 3 and 23 carotenoid compounds and thus occupied variable parts of the species' network (Morrison and Badyaev

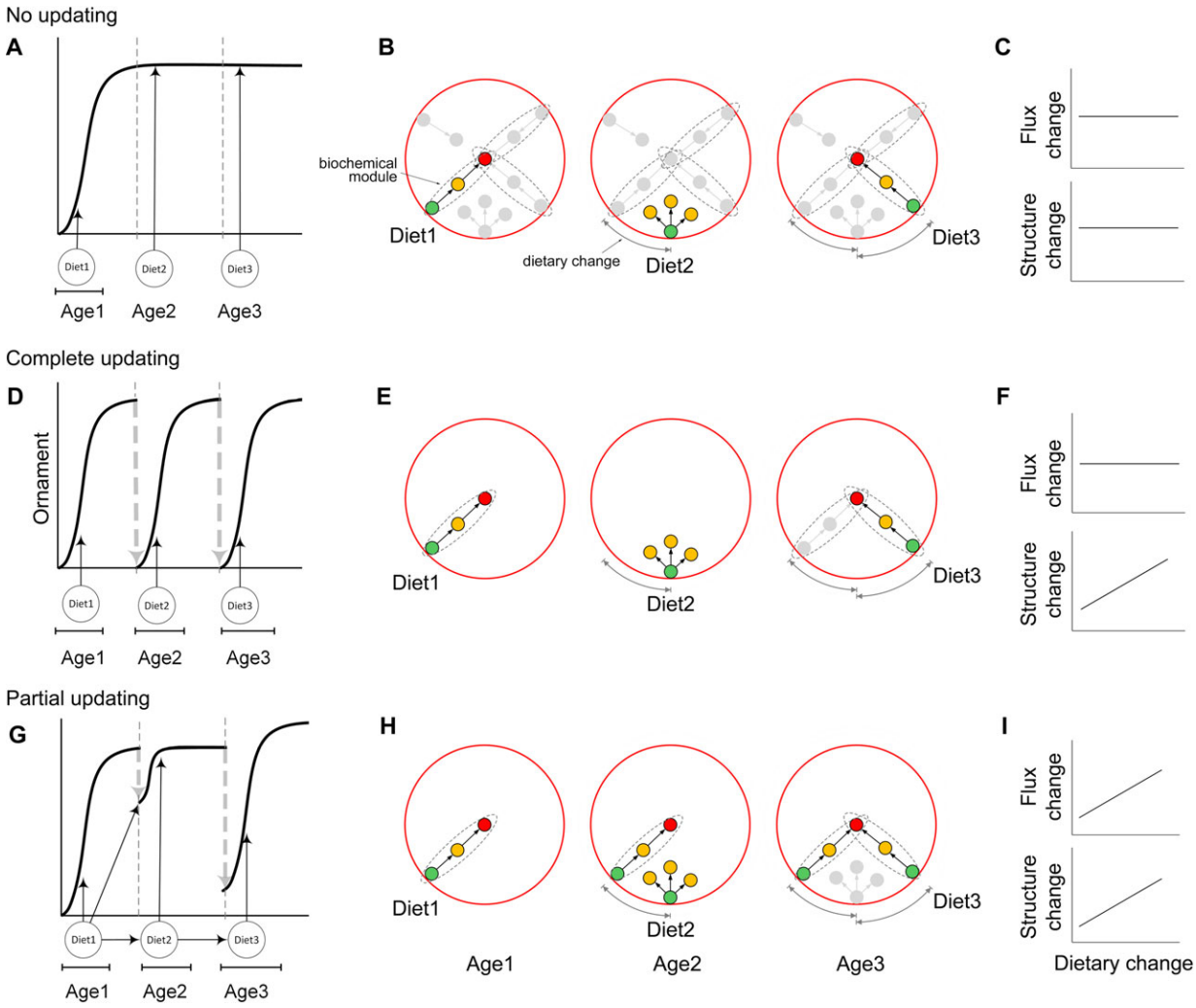

Figure 1. Three scenarios for reiterative development of diet-dependent ornamentation throughout a lifetime. Under the "no updating" scenario (A–C), the ornament production machinery does not change throughout a lifetime and is buffered against changes in dietary inputs, having been set during the initial development in the first molt of life ("sensitive window" shown as bracket, age boundaries shown in vertical dashed lines) or inherited. (B) Distinct dietary carotenoids (light, green nodes) at different ages are utilized by different parts of preexisting biochemical network (enclosed in large red circle, dashed lines enclose biochemical modules). Depending on biochemical network connectivity in the vicinity of encountered dietary carotenoids, some dietary change between consecutive molts (double-headed arrows outside of red circle) result in production of distinct derived carotenoids (light, yellow nodes), whereas others result in production of carotenoids that can be redundantly reached from different dietary starting points (dark, red nodes). (C) Under this scenario, dietary change between molts is not associated with change in either flux (because different pathways are used in each molt) or network structure (which is constant throughout life). Under the "complete updating" scenario (D–F), the ornament production depends on the dietary input during the nearest molt (i.e., "sensitive window"–brackets), without the influence of the prior ornament states. (E) Depending on biochemical connectivity in the vicinity of dietary inputs, different parts of network are utilized. (F) Under this scenario, dietary change is not associated with change in flux between the molts (because different parts of network are used in molt episodes), but is associated with utilization of a different part of the network (different expressed structure). Under the "partial updating" scenario (G–I), the ornament is affected by interaction of both prior states and changing current input. In addition to affecting the current state of the ornament (vertical arrows), previous diet affects the efficiency of metabolism of the subsequent diet (angled arrow) and ability to obtain that diet (horizontal arrows). (H) When previous exposure to dietary carotenoids has a priming effect on metabolism of carotenoids in subsequent molts, it is partially retained, such that a greater portion of metabolic space is occupied with age or experience. (I) Under this scenario, larger dietary change is associated with lesser flux similarity between ages (because of the priming effect of preceding dietary carotenoids) and utilization of different parts of network structure. Because the network structure is known for the species, testing distinct predictions in age- and experience-related changes in network utilization and flux enables separation of these three scenarios that otherwise make overlapping predictions for ornamental change in relation to dietary change (because, under all scenarios, ornament change can be masked by carotenoids derived through biochemically redundant pathways).

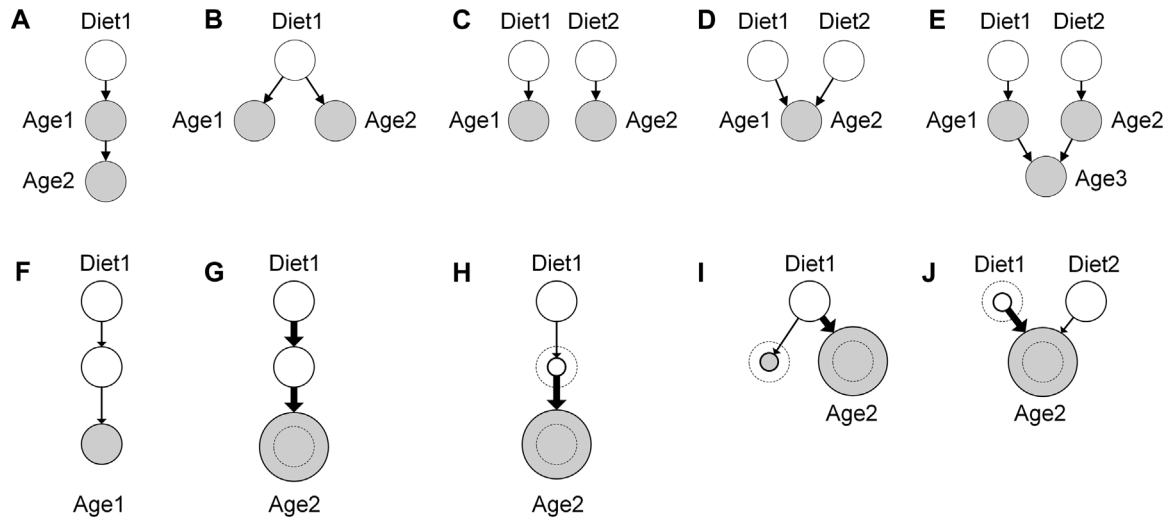

Figure 2. Age-related changes in the biochemical network underlying the production of carotenoid-based sexual ornamentation. Compounds are shown as circles (shaded: derived carotenoids, open: their dietary carotenoid precursors) connected by reactions (arrows). Upper row shows possible changes in the structure of biochemical networks, lower row shows change in the flux of the biochemical network from the preceding to the subsequent age. (A) Elongation of enzymatic pathways, (B) the use of different reactions from the same starting point (from either dietary, as shown, or derived), (C) the use of different biochemical modules, each with its own dietary starting point, (D) dietary redundancy that supports production of the same compound from age-specific dietary compounds, (E) biochemical redundancy that enables production of a derived compound from precursors produced at earlier ages. (F) The flux of a biochemical network of the preceding age (Age1) shown as reference, (G) proportional increase in flux along enzymatic pathways (thicker arrows) leading to greater accumulation of the derived carotenoid (end product, dashed line shows previous state), (H) disproportional changes in flux along an enzymatic pathway leading to corresponding changes in relative concentration of compounds along the pathway (either at the end of the pathway, as shown, or at any other location on the pathway), (I) disproportionate partitioning of substrate between products, and (J) changes in the proportion of dietary precursors between ages.

2017). We obtained concentrations (μg/g of pigmented feathers) for 16 carotenoids deposited in the plumage. For each sample, we constructed a metabolic network by mapping carotenoids identified in that sample onto the house finch complete enzymatic network (Fig. S1, protocol in Morrison and Badyaev 2017). This resulted in $n = 888$ metabolic networks (three ornaments for 74 individuals for four consecutive ages). Feathers within each ornamental area were combined for carotenoid extraction and analyses.

NETWORK STRUCTURE

For each network, we calculated numbers of compounds and reactions, network diameter (the longest minimum distance, in reactions, between any two compounds), average shortest pathway (the average length, in reactions, of the shortest paths between all of the pairs of compounds), average degree (the average of incoming and outgoing reactions per compound). Structural distance between any two networks was calculated as Jaccard distance, J (1-Jaccard similarity), based on the fraction of reactions and compounds shared between the networks. Structural distance was also calculated separately for dietary and derived compounds. *Dietary change* was the Jaccard distance between any two age-specific networks based on the presence of only their dietary carotenoids.

FLUX CHANGE

Network flux is the distribution of compound concentrations across the network. It is therefore affected by both changes in concentrations (caused by enzymatic efficiency or concentration of substrates, Fig. 2F–J) and changes in the structure of biochemical pathways (such as gain or loss of reactions and compounds, Fig. 2A–E) (Basler et al. 2016; Morrison and Badyaev 2016a). Thus, measurements of flux change between ages require a method that does not assume similarity in network structure between ages. This rules out common rank correlation approaches, such as Kendall's Tau or Spearman r, because these approaches compare two ranked lists and measure probability of elements occurring in the same order between them (Zar 1984). Thus, they cannot be used when ranked lists differ in length or composition, such as when some carotenoid compounds are gained or lost between ages. Further, in the rank correlation approaches, the rank position has no effect on the similarity score, such that large and small changes in concentration of compounds between ages have the same similarity scores if they produce identical ranking lists.

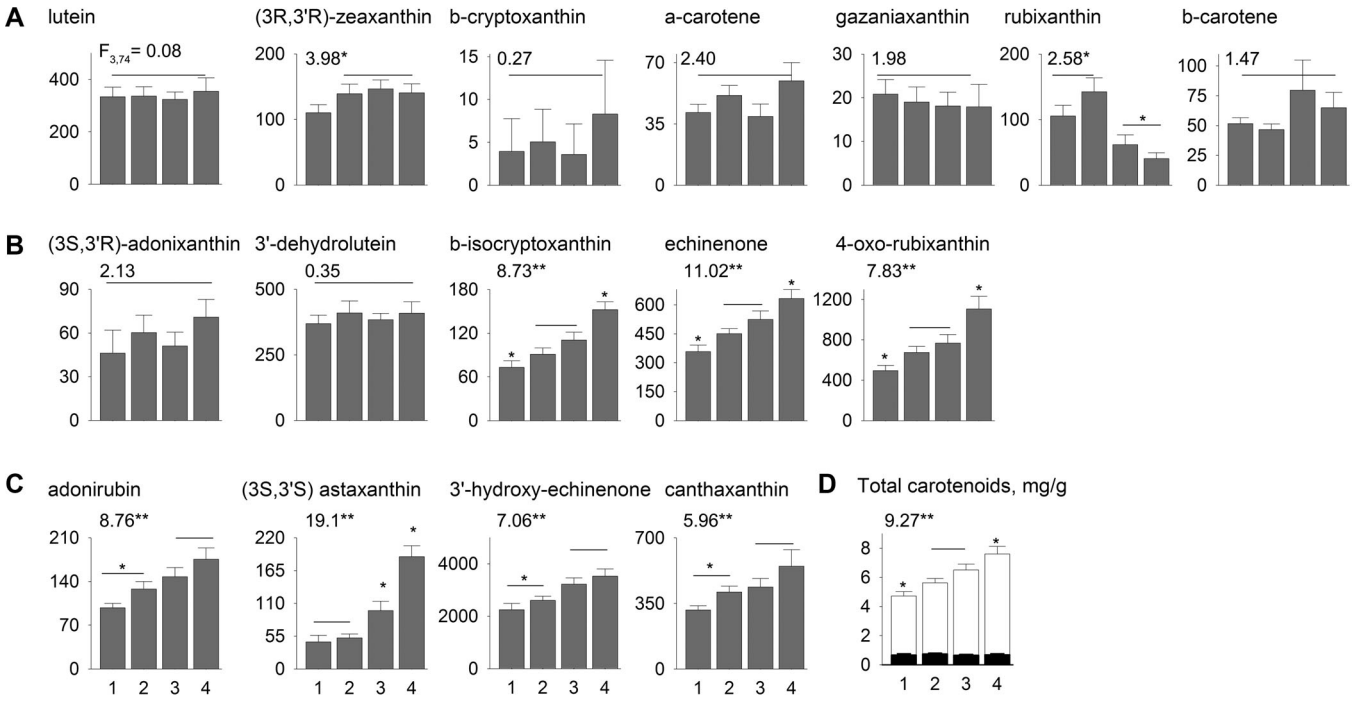

Figure 3. Carotenoid concentration (μg/g of pigmented feather, mean ± SE) in four consecutive ornaments (n = 74 males) starting from the first molt of life. Breast ornament is shown. (A) Dietary carotenoids, (B) carotenoids derived from dietary carotenoids by one enzymatic reaction, (C) carotenoids derived from dietary carotenoids by two or more enzymatic reactions. (D) Total amount of carotenoids (mg/g of pigmented feather). Black bars show dietary carotenoids, white portion of bars—derived carotenoids. Numbers show F values for significance of Age from the repeated measures ANOVA of four consecutive ornaments for every individual. $^{*}P < 0.05$, $^{**}P < 0.01$. Lines connect means that are not different under consensus of the Ryan-Einot-Gabriel-Welsch multiple range and Duncan's multiple range tests.

To overcome these limitations, we used the rank-based overlap (*rbo*) similarity measure that assesses the overlap between any two ranked lists that are dissimilar in length or composition as a proportion of the ranking depth, *k* (Fagin et al. 2003; Wu and Crestani 2003; Webber et al. 2010; Konagurthu and Collier 2013). The method starts with calculation of the overlap at depth *k* and then extends it to cumulative overlap at increasing *k*. The similarity measure is the average of these overlaps and *rbo* measures the expected average overlap based on different depths of comparison of the two lists (see Fig. S3 for example of calculations). *rbo* is a similarity measure that varies from 0 (maximally different flux distribution) to 1 (perfectly proportional flux distribution). 1-*rbo*, used here, is a metric distance (Webber et al. 2010), analogous to Jaccard similarity (*J*) and its metric counterpart—Jaccard distance (1-*J*).

DIETARY BIOCHEMICAL REDUNDANCY

To measure biochemical *dietary redundancy* of a compound, we summed all of the unique noncyclical pathways that link a derived carotenoid with each of the dietary carotenoids. This was done with a breadth-first search algorithm (Moore 1957) and calculations for the redundancy for all house finch carotenoids are in Table S1.

Results

CONSISTENT LIFE-LONG TRENDS IN ORNAMENTATION AND THEIR METABOLIC ORIGINS

Relative concentration of derived carotenoids increased with age, especially in highly metabolized carotenoids, whereas the concentration of dietary carotenoids remained the same, with the exception of a lower concentration of rubixanthin in the last two age groups (Fig. 3). Correspondingly, ornament hue (redness) and saturation increased over the lifetime in all three ornamented areas, but most strongly so during the transition from the first to the second ornament (Fig. 4A and C). Ornamental areas did not change over the lifetime (Fig. 4B).

Individuals increased the size of the metabolic network they occupied throughout their lifetimes, adding more carotenoid compounds (number of nodes, Fig. 5A) and reactions (edges, Fig. 5B). However, these compounds and reactions tended to be added to the existing biochemical modules because neither the network diameter nor the average degree increased over the lifetime (Fig. 5C

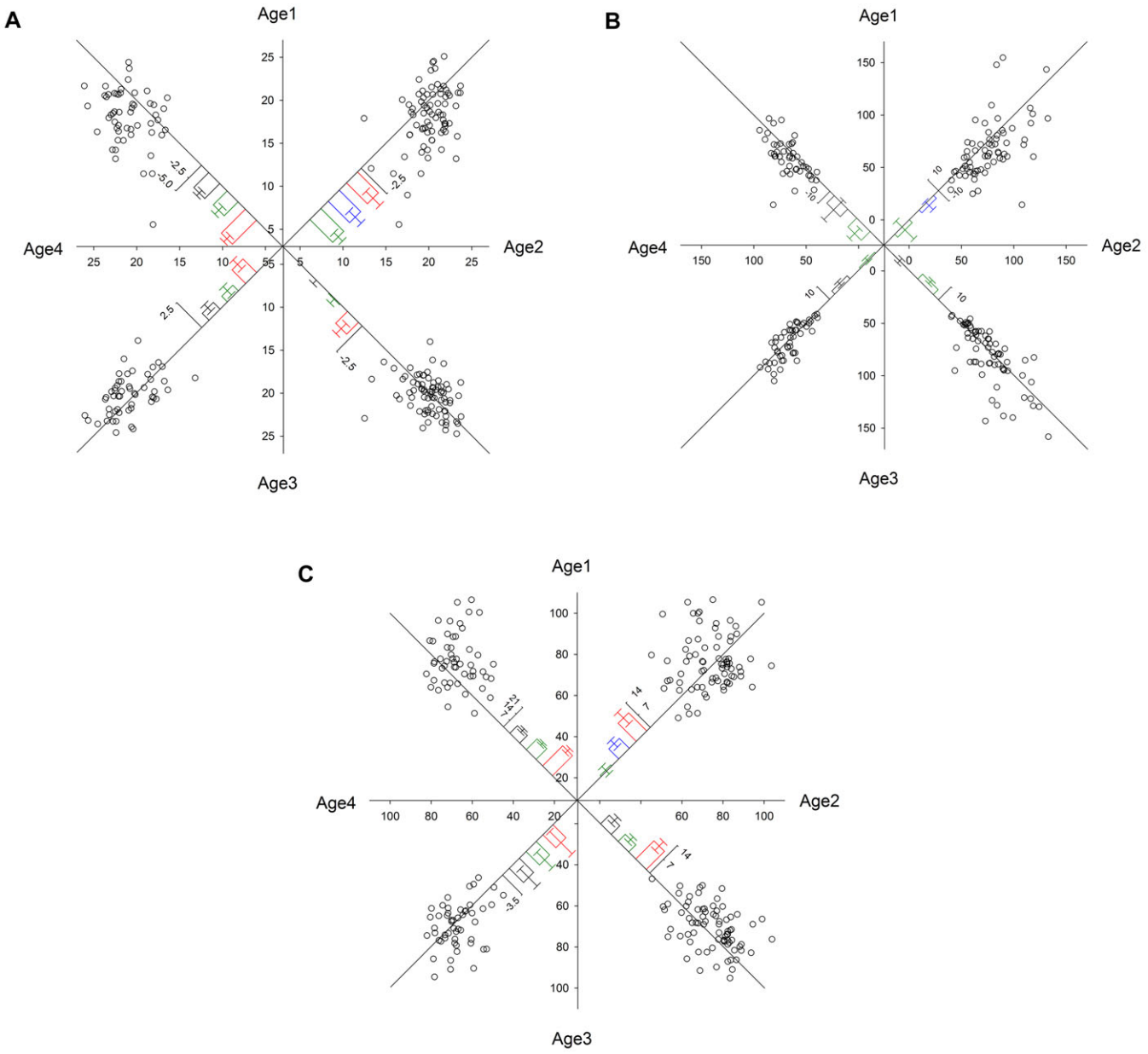

Figure 4. **Changes in ornamentation across four consecutive molts within a lifetime. Points on diagonal in each quartile are not different between the ages (axes). Last quartile shows lifetime change between the first and the fourth molt of life. Points show values for breast ornamentation, bars (mean ± SE) show average change for each pairwise comparison for all ornaments. Bars protrude toward the group with larger values. (A) Hue of ornament (degrees). Bars show average change in the hue of breast (green), crown (blue) and rump (red). (B) Area of ornament (sq. pixels). Bars show average change in area of breast ornament (green) and rump ornament (blue). (C) Intensity (brightness—proportion of white) of ornament (%). Bars show average change in intensity of ornament of breast (green), crown (blue), and rump (red).**

and D). Instead, the network degree decreased over the lifetime, reflecting strictly modular placement of newly added nodes (Fig. 5D). Greater modularity and shorter length of biochemical pathways were associated with greater ornamentation at all ages, except the fourth molt and, at all ages, greater amount of derived carotenoids in feathers led to greater ornamentation (Fig. 6).

VARIABLE USE OF THE METABOLIC NETWORK THROUGHOUT LIFETIME

Individuals varied in utilization of their network over their lifetimes, using different combinations of compounds and reactions as well as modifying the flux distribution among them (Fig. 7A). In transition from the first to the second molt of life, individuals

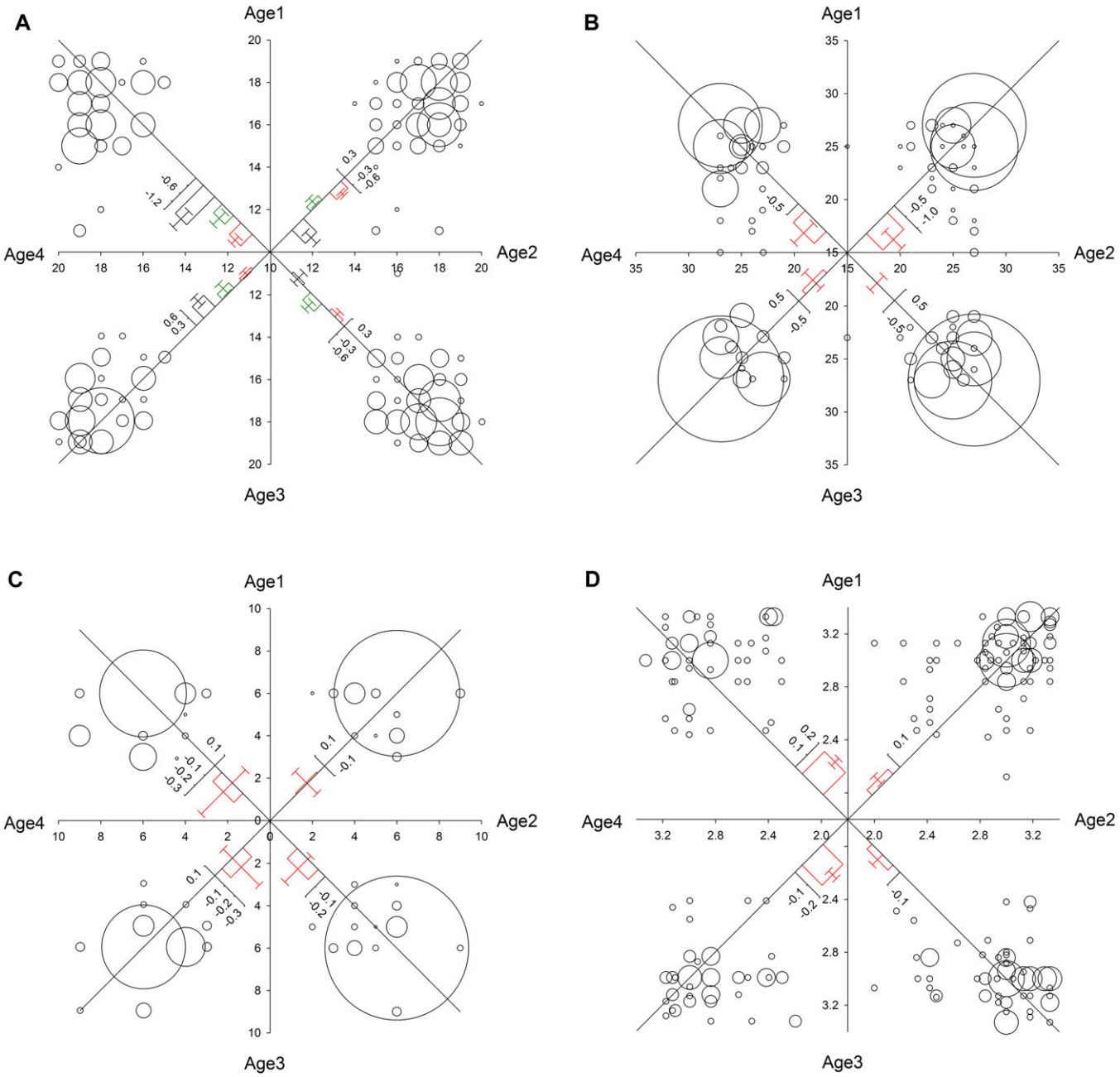

Figure 5. Changes in network structure across four consecutive ornaments within a lifetime. Diameter of points is proportional to the number of individuals with the same values. See legend of Figure 4. (A) Number of network nodes (carotenoid compounds) in each network. Bars show change in total number of carotenoids (black), dietary carotenoids (green), and derived carotenoids (red). (B) Number of network edges (reactions), (C) network diameter (in reactions), and (D) network's average degree (in reactions).

tended to most strongly change the flux distribution, primarily of derived carotenoids (Fig. 7A and B), producing, in the second molt, a greater amount and diversity of them (Fig. 3; Fig. 5A, red bars). In subsequent ornament iterations, individuals mostly changed the network structure, primarily through additions of new dietary carotenoids (Fig. 7A; Fig. 5A, green bars). The diversity of newly encountered dietary carotenoids remained similar across the lifetime (Fig. 7B–D, left column), whereas the diversity of carotenoids derived from them decreased (Fig. 7B–D, right column).

PREVIOUS EXPOSURE TO DIETARY CAROTENOIDS AFFECTS THEIR FLUX IN SUBSEQUENT MOLTS

In individuals transitioning from the first to the second molt, a change in composition of dietary carotenoids (dietary change) had a strong effect on ornamentation, both directly and through

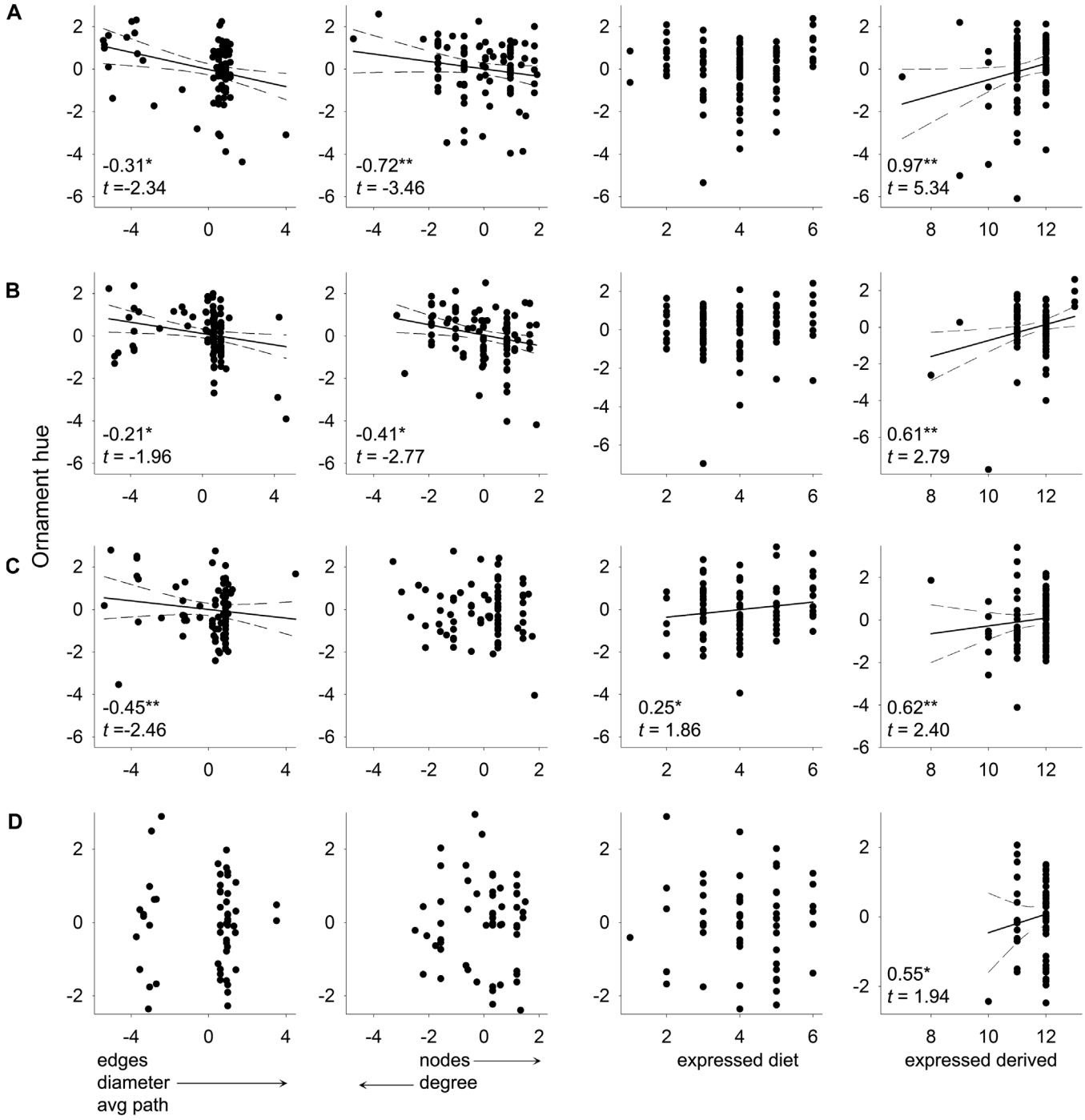

Figure 6. Partial regression plots showing the effect of biochemical network metrics on ornament hue during (A) the first molt of life, (B) the second molt, (C) the third molt, and (D) the forth molt. Ornament hue was Principal Component 1 = 0.58 Breast Hue + 0.57 Crown Hue + 0.58 Rump Hue (80% of variance, eigenvalue $\lambda_1 = 2.41$). Network measures were combined into two principal components (PC) that best captured their correlational structure (left two columns): PC1 = 0.49 Edges (reactions) + 0.55 Network diameter + 0.55 Average path (62% of variance, $\lambda_1 = 3.14$). PC2 = 0.82 Nodes (compounds) – 0.59 Average degree (32% of variance, $\lambda_2 = 1.58$). Right two columns are the number of dietary and derived carotenoids in ornament correspondingly (termed "expressed diet" and "expressed derived"). Regression plot (solid line) with 95% confidence intervals (dashed line) is shown for statistically significant slopes only. Numbers within graph panels are standardized regression coefficients (b_{ST}, in SD) and associated *t*-values. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

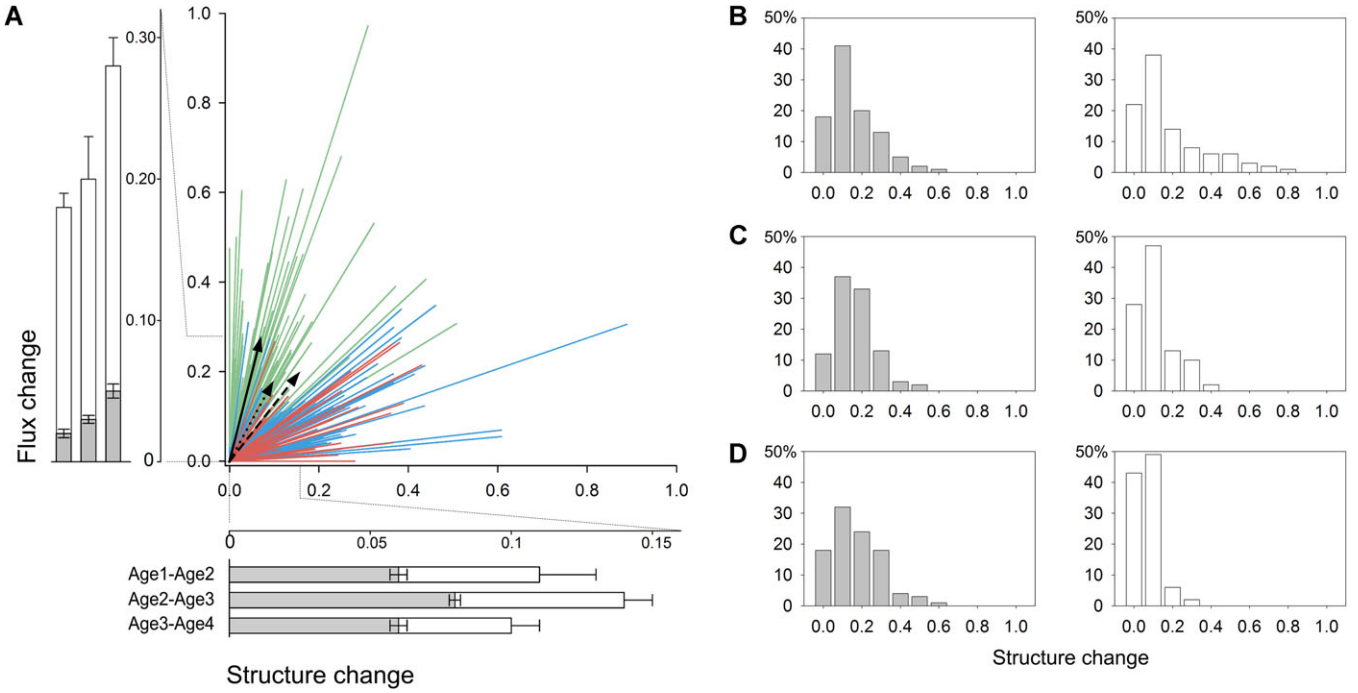

Figure 7. **(A) Changes in structure and flux of biochemical networks of individual house finches ($n = 74$) across four successive ornament changes within a lifetime. Each individual is shown as a vector originating at the previous state of structure and flux (Age_{n-1}) and ending at the next state (Age_n). Green shows individuals changing from Age1 (the first molt of life) to Age2, blue vectors are the same individuals changing from Age2 to Age3, and red vectors—from Age3 to Age4. Many vectors have zero length. *Structure change* is Jaccard distance (1-Jaccard similarity) based on the overlap in use of compounds and reactions between an individual's subsequent ornaments. *Flux change* is a distance (1-*rbo*, see Methods and Fig. S3), based on similarity of flux distribution between the networks of individual's consecutive ornaments. Black vectors show the average change for each age group: Age1→Age2 (solid), Age2→Age3 (dashed), Age3→Age4 (dotted). Bar insets under the axes shows decomposition of these average age-specific vectors in network structure change (*x*-axis) and flux change (*y*-axis) into changes in dietary carotenoids (gray, mean ± SE) and changes in derived carotenoids (white). (B–C) Frequencies of network structure change for dietary carotenoids (gray, left column) and for derived carotenoids (white, right column) throughout the lifetime. (B) Age1 to Age2, (C) Age2 to Age3, and (D) Age3 to Age4.**

modified flux (Fig. 8A and B). In older age classes, ornamentation was increasingly buffered from direct effects of dietary change (Fig. 8C–F), even though the magnitude of dietary change did not change over the lifetime (Fig. 7B–D). In older age classes, dietary change affected ornamentation mostly indirectly, through changes in the flux distribution on the network (Fig. 8C–F).

INCORPORATION OF BIOCHEMICALLY REDUNDANT CAROTENOIDS ENABLES NONDESTRUCTIVE UPDATING OF ORNAMENTS

The buffering of ornamentation from dietary change was due to greater inclusion of biochemically redundant carotenoids which were less affected by dietary change (Fig. 9). Incorporating biochemically redundant carotenoids was largely a function of greater occupancy of the metabolic network: in all ages, individuals that occupied a larger portion of the species' metabolic network, included more biochemically redundant compounds in their active networks and experienced lesser sensitivity of ornamentation to dietary change as a result (Fig. S2, summary in Fig. 10A).

EXPERIENCE LEADS TO BUFFERING FASTER THAN AGE

Greater occupancy of a metabolic network is necessarily linked to experience with dietary change between successive molts because different dietary compounds are utilized by different parts of the metabolic network (Figs. 1 and 7). Older individuals that experienced more of such changes are expected to explore a larger proportion of the network and correspondingly encompass more redundant carotenoids. This is indeed the case, on average (Fig. 10B and C). However, wider cumulative network exploration is, proximately, a function of the magnitude and sequence of dietary changes (Fig. 1), not of age per se; some individuals that experienced large early dietary changes explored more of their network by their second molt than others by the end of their fourth (Fig. 7). Indeed, neither the proportion of the network explored

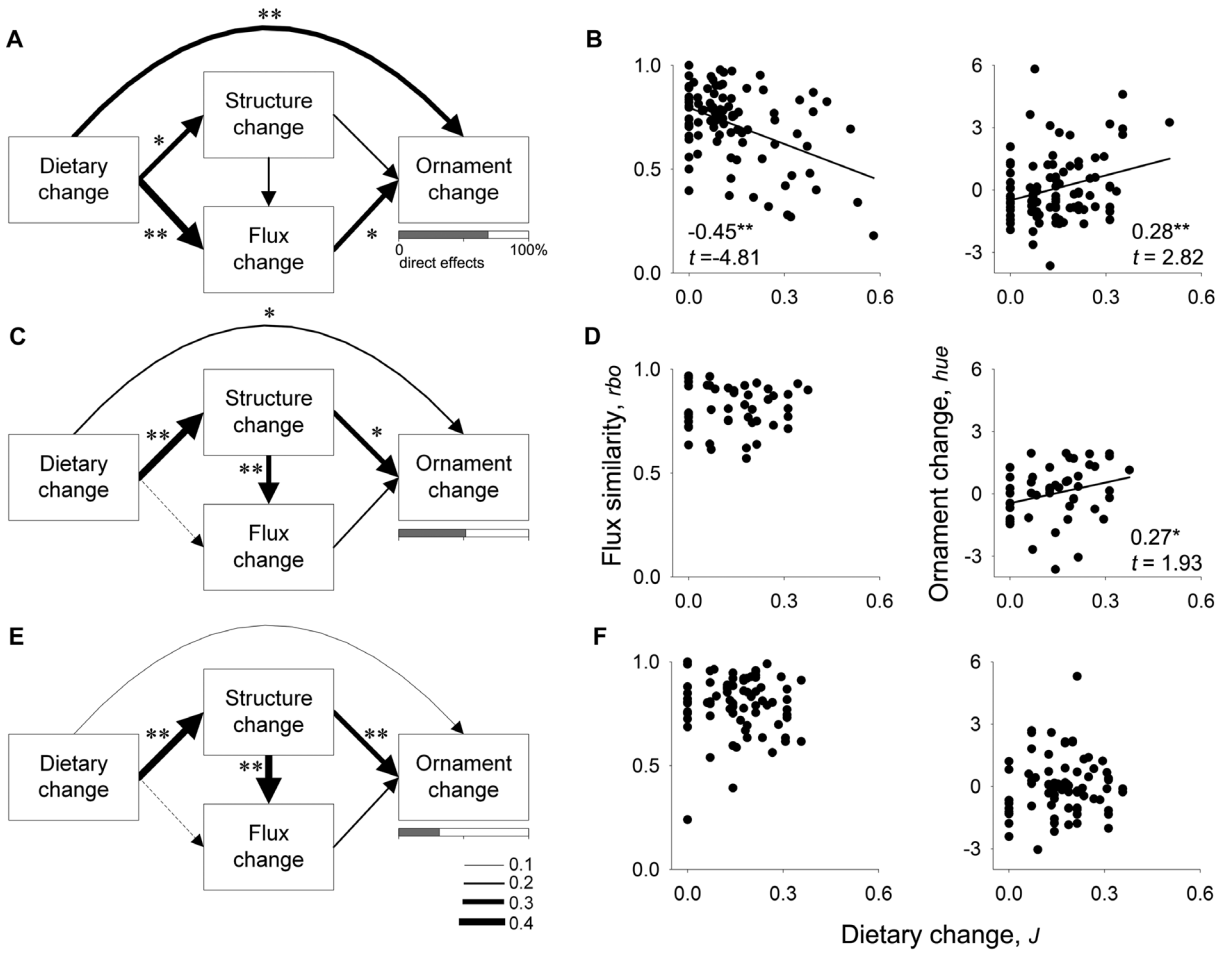

Figure 8. **Direct and indirect effects of dietary change between molts on change in ornament throughout the lifetime. Plots on the left show path diagrams for the effect of dietary change, plots on the right show simple regressions of dietary change on flux similarity and ornament change between subsequent years for (A, B) the first versus the second ornament, (C, D) the second versus the third ornament, (E, F) the third versus the fourth ornament. Bar under the ornament change box (left) is partitioned by the direct effects of dietary change (dark portion) and indirect effects of dietary change (light portion). Arrow thickness is proportional to path coefficients (in standard deviations, SD, of the response variable). Solid arrows indicate positive coefficient, dashed arrow—negative coefficient. Numbers in graphs are standardized regression coefficients (b_{ST}, in SD) $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.**

by individuals within each age group (Fig. S2) nor the magnitude of dietary changes varied across ages (Fig. 7B–D, left column). Because the network structure, dietary change and overall network connectivity were known for each age, we could distinguish the effects of age versus experience on network occupancy and the inclusion of biochemically redundant carotenoids. We found that dietary change was consistently associated with inclusion of biochemically redundant carotenoids across all ages and Age × Dietary change effect was not statistically significant in the repeated measures ANOVA analyses (Fig. 10D). As such, individual experience with dietary change had a greater effect on network occupancy and corresponding inclusion of biochemically redundant compounds and associated robustness of ornamentation than age per se (Fig. 10D).

Discussion

We found that experience-related buffering links evolvability and robustness in carotenoid-metabolizing networks and show that this provides a mechanism for adaptive phenotypic flexibility in resulting traits. Specifically, we report that structural redundancy assures functioning of the network despite fluctuations in environmental inputs, but only when previous use primes a subset of its enzymatic pathways (Fig. 8A–D). This finding causally links form and function in the evolution of phenotypic plasticity–long a goal of evolutionary theory (Osborn 1896; Baldwin 1902; Schmalhausen 1938; Gause 1940; West-Eberhard 1989; Schlichting and Smith 2002; Piersma and Drent 2003; Gerhart and Kirschner 2007; Palmer 2012; Newman 2014). On a population level, a diversity of dietary inputs among individuals activated

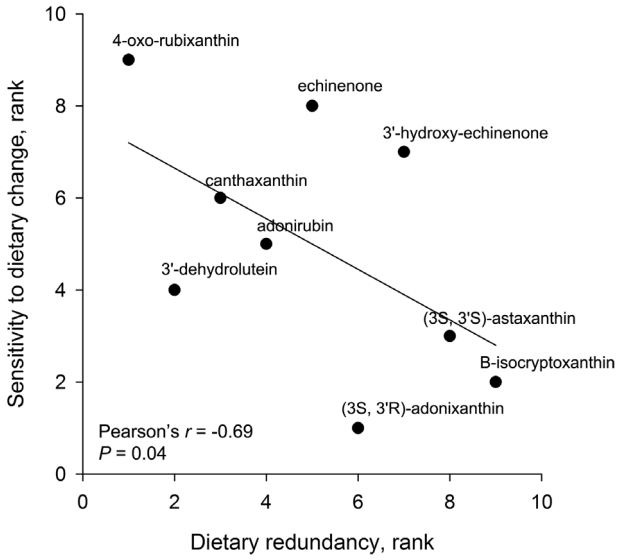

Figure 9. Biochemically redundant carotenoids are less sensitive to between-molt changes in dietary precursors. Sensitivity to dietary change (*y*-axis) is ranks of regression slope of compound concentration change (between age changes in Fig. 3) on dietary change (*J*). Here, the sensitivity to dietary change is plotted against the ranks of dietary biochemical redundancy (number of biochemical pathways by which each compound can be reached from distinct dietary precursors, from Table S1).

different biochemical modules in the network, facilitating network maintenance even though no single individual utilizes the entire metabolic network space within a lifetime (Fig. 10B). Over evolutionary time, such redundancy in network structure necessarily retains the diversity of network use of prior generations, resulting in a structure that potentiates adaptations to multiple environments simultaneously, both within and between generations (Maynard Smith 1970; Edelman and Gally 2001; Promislow 2005; Lenski et al. 2006; Espinosa-Soto et al. 2011; Wagner 2011). Importantly, the priming requirement for the activation of redundant biochemical pathways allows the coexistence of specialization and versatility within the network. For example, throughout a lifetime most individuals pruned and streamlined their networks (Fig. 5), especially when encountering similar dietary inputs between molts, and this resulted in greater channeling of derived carotenoids into ornamentation (Figs. 3B and C and 4A). However, the presence of biochemically redundant carotenoids at the intersection of diet-specific biochemical modules allows a rapid switch between them, enabling exploration of distinct biochemical phenotypes without detrimental effects on ornamentation (Fig. 8E). Within a generation, greater prior experience further lowers the cost of accommodating novel environmental inputs–a general principle in learning, immunity, and development. These findings give two insights into the evolution of phenotypic plasticity.

First, they provide a mechanistic basis for often documented age-related phenotypic plasticity. Modeling studies have predicted less phenotypic adjustment at earlier ages until sufficient information about the environment has been accumulated to warrant change and later in life where the benefits of phenotypic adjustment progressively diminish with declining reproductive value and weaker selection on older individuals (Fischer et al. 2014; Fawcett and Frankenhuis 2015). Further, both accumulated experience and history of past phenotypic adjustments are thought to bias phenotypic adjustment in older individuals and increase the time required to arrive to appropriate solutions (Frankenhuis and Panchanathan 2011; Stamps and Krishnan 2014, 2017). Patterns of age-related genetic variance typically mirror these patterns, with inherited effects dominating at early and late ages, and acquired, learned and compensatory strategies prevailing at intermediate ages (Cheverud et al. 1983; Charmantier et al. 2006; Hegyi et al. 2006; Evans et al. 2011). Our study reveals the mechanistic basis for these predictions—increasing experience was structurally linked to greater plasticity potential—which also provided a mechanism for the commonly observed correlation between early experience and subsequent plasticity in neurological and physiological studies (reviewed in Piersma and van Gils 2010; Bateson et al. 2014; Beaman et al. 2016; Duckworth et al. 2018). In addition, we showed how accumulating experience mechanistically leads to emergent buffering of trait development and thus progressively lesser costs of phenotypic adjustment (Figs. 8 and S2). Adjustment of a trait at early age classes was less buffered (and thus presumably costlier), however this cost was proportional to the lesser cost of subsequent adjustments; indeed, this early cost is an essential building block of the subsequent plasticity (Fig. 8A). This finding emphasizes the importance of considering lifetime fitness benefits of plasticity, because mechanisms of phenotypic adjustment at different life stages are unlikely to be independent (Nettle and Bateson 2015; English et al. 2016).

In older age classes, accumulation of prior experience (and thus functional activation of pathways, Fig. 10) can bias the production of some compounds at the expense of others, but only in individuals that were repeatedly exposed to an identical and small subset of dietary precursors, which is rare under natural conditions (Fig. 7). Even small changes in dietary inputs that accumulated during a lifetime were enough to lead to rapid acquisition of versatility in metabolic network (Fig. 10C and D) and to the resulting buffering of ornamentation (Figs. 8 and S2). This enabled older individuals to maintain the same ornamentation across a range of environmental inputs; however this was not due to the lack of response to dietary fluctuations, but due to greater masking of them. Although all age classes experienced the same level of environmental variability and showed extensive modulation of the structure and flux of utilized metabolic networks as they accommodated this variability (Fig. 7), they differed in

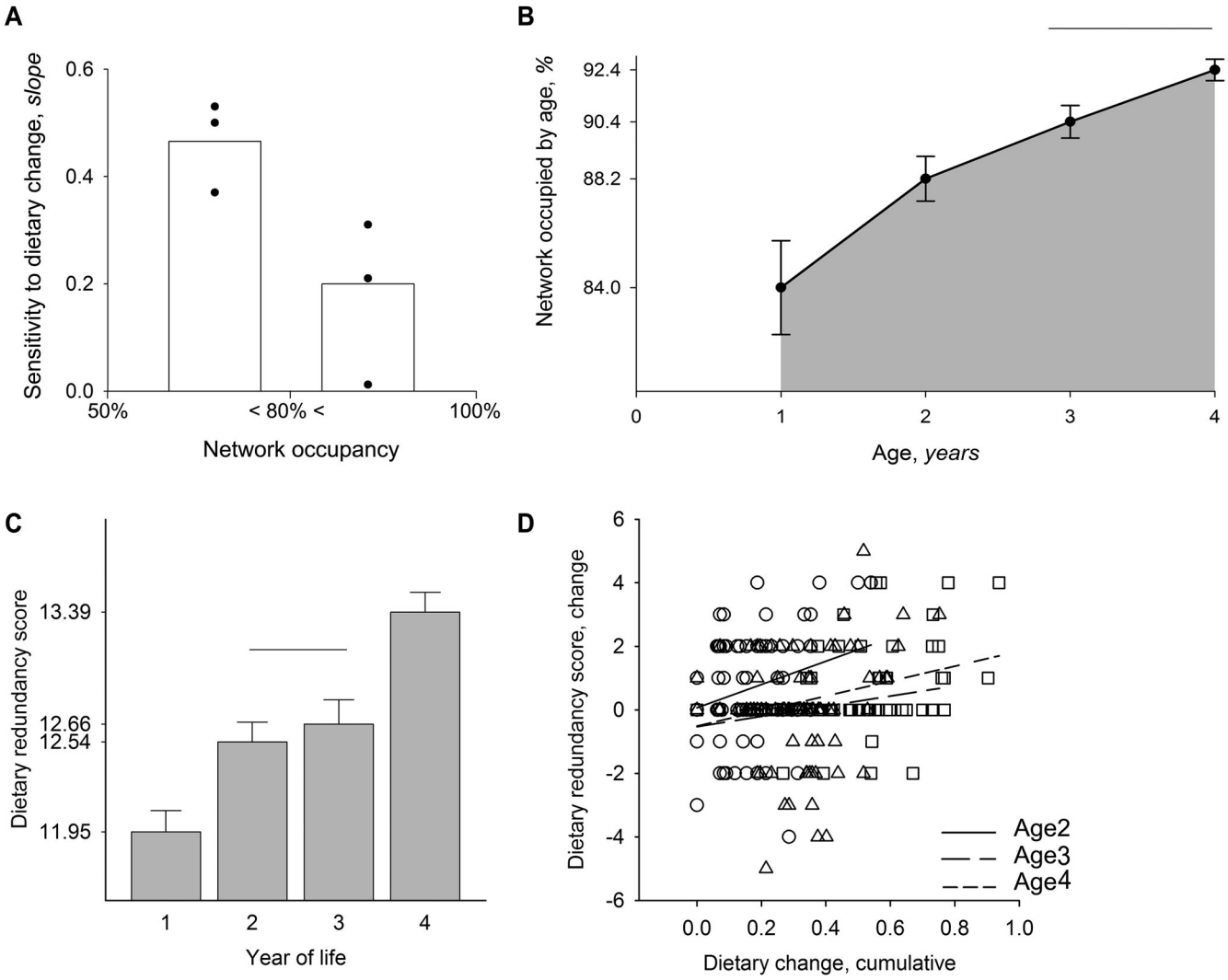

Figure 10. **(A) In all ages, greater cumulative occupancy of the network was associated with lower sensitivity of ornamentation to dietary change (summary of data from Fig. S2). *y*-axis is regression coefficient (b_{ST}) for the overall regression of dietary change on ornament change for the two classes of network occupancy (<80% and 80%<; network is shown in Fig. S1). Data points are regression coefficients for each age class and network occupancy group (from Fig. S2). (B) Percent of the full species network [observed compounds/total number of compounds (Fig. S1)] occupied by each age (mean ± SE). Line spans similar means. (C) Dietary redundancy score (count of biochemically redundant compounds, mean ± SE) throughout the lifetime. Line spans similar means. (D) Change in dietary redundancy score in relation to the cumulative dietary change [Age2 (circles, solid line): $b_{ST} = 0.27$, $t = 3.05$, $P < 0.01$, Age3 (triangles, long dash): $b_{ST} = 0.16$, $t = 1.89$, $P = 0.10$, Age4 (squares, short dash): $b_{ST} = 0.38$, $t = 3.63$, $P < 0.01$]. Repeated measures ANOVA (dietary change: $F = 3.84$, $P = 0.05$, age: $F = 15.65$, $P < 0.001$).**

the ability of their metabolic network to absorb and compensate for it (Fig. 8). This cautions against using phenotypic plasticity expressed at the level of the trait as a sole metric of phenotypic adjustment. Clearly, in our system, the focal point of a trait's phenotypic adjustment is shifting upstream in its' developmental hierarchy as individuals accumulate environmental experience.

Second, the role of pathway redundancy in reconciling versatility and robustness within a generation, remarkably mirrors its role on macroevolutionary time scales where evolutionary gains and losses of biochemically redundant carotenoids were closely associated with the rate of evolutionary diversification in carotenoid-based traits (Badyaev et al. 2015) and with maintenance of long-term evolutionary trends in organismal integration of these traits (Higginson et al. 2016). The link between functional use and metabolic robustness found here (Fig. 10), coevolution of biochemically redundant carotenoids and integument structure (Badyaev et al. 2017), as well as evolutionary linkage between metabolic elongation, biochemical redundancy

and selective expression of carotenoids (Higginson et al. 2016), strongly suggest that connectivity of the underlying network provides a shared structural basis for processes operating at vastly different evolutionary scales—from phenotypic adjustment to macroevolutionary trends (Gavrilets 2004; Wagner 2011). It is tempting to speculate that biochemical pathway redundancy, or more generally, the structure of a metabolic network, evolves as a consequence of the most repeatable or efficient flux, when consistent functional priming within generation results in structural redundancy of primed compounds on evolutionary scales. Although this explanation is routinely advanced for evolution of metabolic networks in other systems (Stelling et al. 2002; Almaas et al. 2005; Ciliberti et al. 2007; Kim et al. 2007; Basler et al. 2016; Eloundou-Mbebi et al. 2016), it is unlikely for carotenoid-producing networks in birds, given the ancient origin and extraordinary conservation of these networks throughout metazoan evolution. Instead, we suggest that the key structural property of carotenoid networks—biochemical pathway redundancy—allowed for continuity of evolutionary processes operating at vastly different time scales in the species diversifying on this network, empirically illustrating that both robustness and evolvability are necessary conditions for continuous evolution (Maynard Smith 1970; Gavrilets 2004; Wagner 2005; Badyaev 2018).

AUTHOR CONTRIBUTIONS

AVB designed the study and supervised field and laboratory data collection. ESM supervised laboratory work, network and digital image analyses, and organized the datasets. Both authors designed analytical tools, collected and analyzed the data, and wrote and edited the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. The house finch carotenoid synthesis network.
Fig. S2. Regression plots of the effect of dietary change on ornament change in two classes of cumulative network occupancy.
Fig. S3. A worked example of using rank-based overlap (*rbo*) to calculate flux change in carotenoid compounds.
Table S1. Components of biochemical redundancy for house finch plumage carotenoids.