



Understanding stoichiometric constraints on growth using resource use efficiency imbalances

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Contributed by James J. Elser; received October 30, 2023; accepted March 15, 2024; reviewed by James M. Hood and E. Litchman

Growth is a function of the net accrual of resources by an organism. Energy and elemental contents of organisms are dynamically linked through their uptake and allocation to biomass production, yet we lack a full understanding of how these dynamics regulate growth rate. Here, we develop a multivariate imbalance framework, the growth efficiency hypothesis, linking organismal resource contents to growth and metabolic use efficiencies, and demonstrate its effectiveness in predicting consumer growth rates under elemental and food quantity limitation. The relative proportions of carbon (%C), nitrogen (%N), phosphorus (%P), and adenosine triphosphate (%ATP) in consumers differed markedly across resource limitation treatments. Differences in their resource composition were linked to systematic changes in stoichiometric use efficiencies, which served to maintain relatively consistent relationships between elemental and ATP content in consumer tissues and optimize biomass production. Overall, these adjustments were quantitatively linked to growth, enabling highly accurate predictions of consumer growth rates.

ecological stoichiometry | nutritional physiology | carbon | nitrogen | phosphorus

The ability to infer and predict dynamic biological processes from limited observations is of great theoretical and practical appeal. Resource limitation of growth rate, for example, is a key process of interest in physiological, ecological, and agricultural studies (1, 2). To grow, organisms must acquire energy and elements (along with other essential molecules, e.g., fatty acids and vitamins for certain taxa) from their surroundings and assimilate them interactively using conserved metabolic pathways (3–5). In doing so, the relative proportions of these constituents in their tissues are altered in a stoichiometric manner dictated by resource supply rates and ratios (6, 7) as well as the organism's evolutionarily derived capacities for their uptake and use for growth (3, 8, 9). The theory of Ecological Stoichiometry provides a framework for studying these dynamics by examining how the proportional balances of energy and elements shape interactions between organisms and the environment (10). Here, we develop an integrated stoichiometric imbalance approach to predict growth rates of organisms across different resource environments and better understand the multivariate nature of resource limitation.

Connections among Organismal Resource Content, Use Efficiency, and Growth Rate.

Compositional ratios expressed as the amount of a resource (i) in an organism (q_i) as a percentage of its total biomass (M)

$$\frac{q_i}{M} \times 100 = \% q_i$$

serve as the foundation of stoichiometric theory, as they allow for comparisons of the relative amounts of resources within and among organisms and between organisms and the environment (10). Because biomass is composed of multiple resources, stoichiometric theory predicts that imbalanced environmental resource supplies will reduce the biomass production efficiency of an organism, keeping it from growing at its maximum possible rate (11–13). These dynamics arise from the distinct resource requirements of biomolecular synthesis that directly link relative growth rates and use efficiencies of organisms to their multivariate resource composition (7, 14, 15).

The resource composition of an organism reflects the net balance of environmental supplies and organismal uptake and use of these resources. Despite the complexity of metabolism, biomass production is primarily a function of carbon (C), nitrogen (N), phosphorus (P), and energy allocation toward the synthesis and use of ribosomes and proteins (12, 16, 17). These processes are stoichiometrically tied to growth, as rapid growth leads to highly constrained and relatively low cellular N:P ratios (18) due to the disproportionately high demands for P allocation to ribosomal RNA to support elevated rates

Significance

Organisms interact with their environments through the exchange of elements and energy. However, predicting the effects of insufficient supplies of these resources on organismal growth has been a longstanding challenge. To this end, we developed a conceptual framework, the growth efficiency hypothesis, which posits strong mechanistic relationships among organismal resource contents, use efficiencies, and growth rate. We tested this hypothesis by exposing consumers to multiple forms of resource limitation, which resulted in unique differences in their resource composition. These differences reflected physiological changes serving to optimize resource use efficiencies and were used to generate accurate predictions of consumer growth rate. Our findings demonstrate the growth efficiency hypothesis to be a powerful framework for understanding the multivariate nature of resource limitation.

Author contributions: J.J.E. and P.D.J. designed research; C.P. and T.P. performed research; C.P. and T.P. analyzed data; and C.P., T.P., J.J.E., and P.D.J. wrote the paper.

Reviewers: J.M.H., The Ohio State University; and E.L. Carnegie Institution for Science.

The authors declare no competing interest.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2319022121/-DCSupplemental>.

Published April 29, 2024.

of polypeptide synthesis (8, 12). Carbon (C) is less variable than N and P in organisms and is thus commonly used as a proxy of both total biomass and energy content (10, 19, 20), as organic molecules are used to build cellular structures and oxidized to produce higher energy molecules such as adenosine triphosphate (ATP). In addition to powering metabolic reactions, ATP serves as a “Goldilocks molecule,” supporting protein function at high ATP concentrations by inhibiting the formation of protein aggregates in the cytoplasm (21, 22) but reducing protein synthesis rates when levels become too high due to ATP chelation of metals essential for ribosome stability and functioning (23, 24). Due to its thermodynamic and chemical properties, ATP plays a central role in growth regulation and is thus a natural candidate for inclusion in stoichiometric models linking organismal growth rate to resource composition and biochemical allocations (12).

Insufficient environmental supplies of energy and elements elicit unique physiological adjustments in organismal uptake, assimilation, turnover, and elimination of these resources (25, 26). Combined, these changes tend to increase the use efficiencies of limiting resources at the cost of reduced growth rate (27, 28) and altered use efficiencies of other resources (29). Because it is impractical to simultaneously measure all of these dynamics for multiple resources, integrated metrics such as production use efficiency ratios were developed to estimate use efficiencies as the mass-specific growth rate of an organism per unit of q_i allocated to biomass (30):

$$\frac{\left(\frac{\ln(M_{\text{final}}) - \ln(M_{\text{initial}})}{\text{time}}\right)}{q_i}$$

Specific growth rates (31) are often difficult to measure in the field, however, as they require at least two estimates of biomass over time. We propose that stoichiometric relative use efficiency ratios may alternately be formulated as the natural log-transformed biomass of an organism per unit of relative q_i

$$\text{Relative Use Efficiency (RUE)} = \frac{\ln M}{\% q_i}$$

and that the use efficiency of a resource-limited organism may be estimated through comparisons to maximally growing “optimal reference” organisms ($\text{RUE}/\text{RUE}^{\text{optimal}}$) of the same age or mass. By examining relative use efficiency ratios for multiple components (i.e., C, N, P, and ATP), we can gain a better understanding of the multivariate physiological adjustments and tradeoffs in resource use efficiencies for biomass production resulting from imbalanced environmental resource supplies.

Use efficiency ratios may also be estimated at a single time point as the reciprocal of organismal resource content

$$\frac{M}{q_i}$$

reflecting the total amount of biomass (or yield) of an organism per unit of q_i allocated to biomass. While simple to measure, the utility of these ratios as use efficiency metrics has long been questioned (1), leading to the development of a variety of metrics designed for specific field applications (32). To predict specific growth rates, we developed stoichiometric absolute use efficiency ratios representing the total amount of biomass accumulated per unit of relative q_i at a given moment in time

$$\text{Absolute Use Efficiency (AUE)} = \frac{M}{\% q_i}$$

To grow maximally (μ_m), the metabolic efficiencies of RNA, protein, and ATP synthesis and use must be optimized (3, 12, 16), which should be reflected in a constrained set of optimal use efficiency ratios for C, N, P, and ATP

$$\mu_m \approx \left(\text{AUE}_C^{\text{optimal}}, \text{AUE}_N^{\text{optimal}}, \text{AUE}_P^{\text{optimal}}, \text{AUE}_{\text{ATP}}^{\text{optimal}} \right).$$

Given the systems-level connections between these currencies, we posit that under resource limitation, organisms will alter their use efficiencies in concert so that their realized growth rate (μ) is equal to

$$\mu = \mu_m \left[1 - \alpha \sum_{i \in \{C, P, N, \text{ATP}\}} \left(\frac{| \text{AUE}_i^{\text{optimal}} - \text{AUE}_i |}{\text{average}(\text{AUE}_i^{\text{optimal}}, \text{AUE}_i)} \right) \times 100 \right],$$

where α is a proportional constant > 0 (note that the numerator of the AUE ratio is an absolute value; see *SI Appendix, Text S1* for a full derivation). If so, we should be able to estimate μ for resource-limited organisms as the total %difference between their absolute use efficiency ratios and those of optimal reference organisms growing at maximal rates.

We define these comparisons as absolute use efficiency imbalances, which combine the mechanistic insights of stoichiometric theory (10) with the predictive power of optimal reference balance approaches (33, 34). Similar comparisons of resource contents and relative use efficiencies of resource-limited organisms to reference organisms (defined as resource imbalances and relative use efficiency imbalances, respectively) reflect underlying changes in cellular growth processes and physiological tradeoffs induced by different forms of resource limitation. Together, this imbalance framework extends the stoichiometric concept of resource limitation derived from Liebig’s Law (2) to include multiple resources. It also proposes that organismal growth is constrained not only by multivariate resource supply imbalances but also by the interconnected physiological adjustments of organisms to these imbalances. Like the growth rate hypothesis, which posits a tripartite coupling among organismal growth, biochemical allocations, and elemental composition (8), the integrated nature of these imbalance metrics reflects a close coupling of organismal resource contents, use efficiencies, and growth rate thus forming a complementary growth efficiency hypothesis. To test this hypothesis, we raised a model organism, the consumer *Daphnia pulex*, under factorial manipulations of high quality [balanced N and P (NP)], N-limited (LN), and P-limited (LP) diet stoichiometry at high and low food concentrations of each diet (HF and LF, respectively) and quantified resulting differences in their mass-specific growth rates as well as their C, N, P, and ATP contents.

Results and Discussion

Daphnid growth was reduced under all forms of limitation relative to animals growing at near maximal rates on high quality, high food quantity diets (Fig. 1A). By comparing the proportions of elements and ATP in these reference animals [C:N:P:ATP = 90:17:3:1 (mass) and 3850:611:48:1 (mol)] to those of animals in resource-limited treatments, we can see how resource limitation differentially affects growth by altering the proportions of these currencies in organismal tissues by the law of the minimum (2), N-limitation under high food concentrations impaired growth, and N was the only imbalanced resource relative to reference animals. However, for all other treatments, we

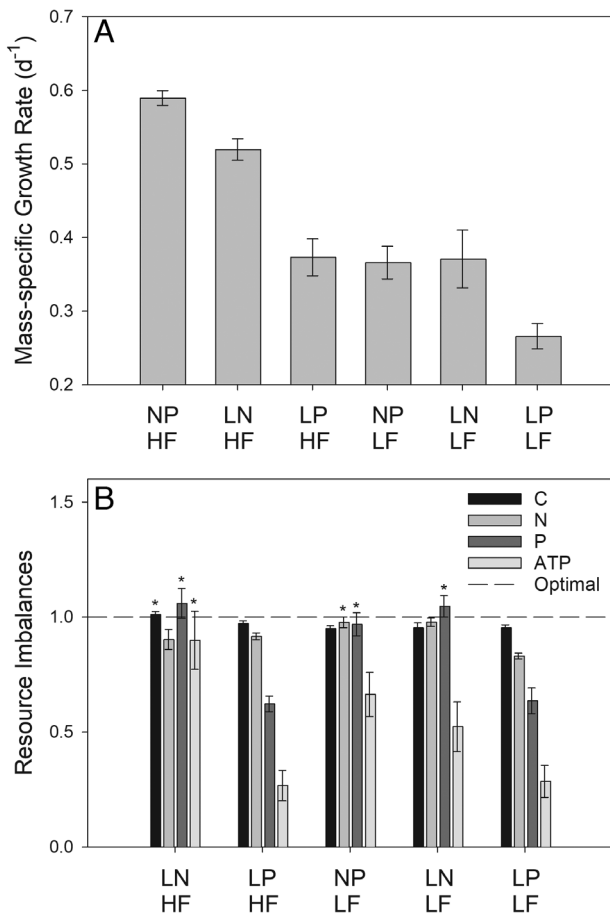


Fig. 1. Effects of diet quantity and quality on *Daphnia* growth rate, elemental composition, and ATP content. (A) Resource limitation reduced mass-specific growth rates compared to optimally growing animals fed high food quantities (HF) of N and P balanced (NP) food ($P < 0.001$). (B) Elemental and ATP contents of resource limited animals are reported relative to NPHF animals and are considered balanced (*) where 95% CIs overlap 1. Other abbreviations include low food quantity (LF), low nitrogen (LN), and low P (LP).

documented unique multivariate shifts in *Daphnia* body composition, suggesting that compensatory metabolic responses to resource limitation altered the proportional balances of all resources simultaneously. These multivariate dynamics are inconsistent with the law of the minimum (35, 36) and highlight the need for extending the concept of resource limitation beyond one or two resources (i.e., colimitation) by acknowledging that organisms require properly balanced supplies of all essential resources to grow optimally (37).

While originally developed as a multivariate framework (13), the stoichiometric study of growth has largely focused on a single element, P (17). The power of P content to predict growth responses, however, was moderate in our study compared to other resources (R^2 : %C-0.77, %N-0.30, %P-0.39, %ATP-0.81; *SI Appendix, Fig. S2*). Instead, the combined proportion of C, N, and P (i.e., %C + %N + %P, referred to hereafter as $\Sigma\%CNP$) was the simplest resource model for predicting daphnid growth rates (Fig. 2A). This result suggests that elemental composition alone can be an accurate predictor of growth rate (*SI Appendix, Fig. S3*), reinforcing recent work showing a consistent linear coupling of elements with growth rate across *Daphnia* species and ontogenic stages (31). The coupling can be attributed to the elevated demands for C, N, and P relative to other elements for biomolecular synthesis and to the strong relationship between $\Sigma\%CNP$ and %ATP contents across resource limitation treatments (Fig. 2B). A relatively consistent $\Sigma\%CNP$

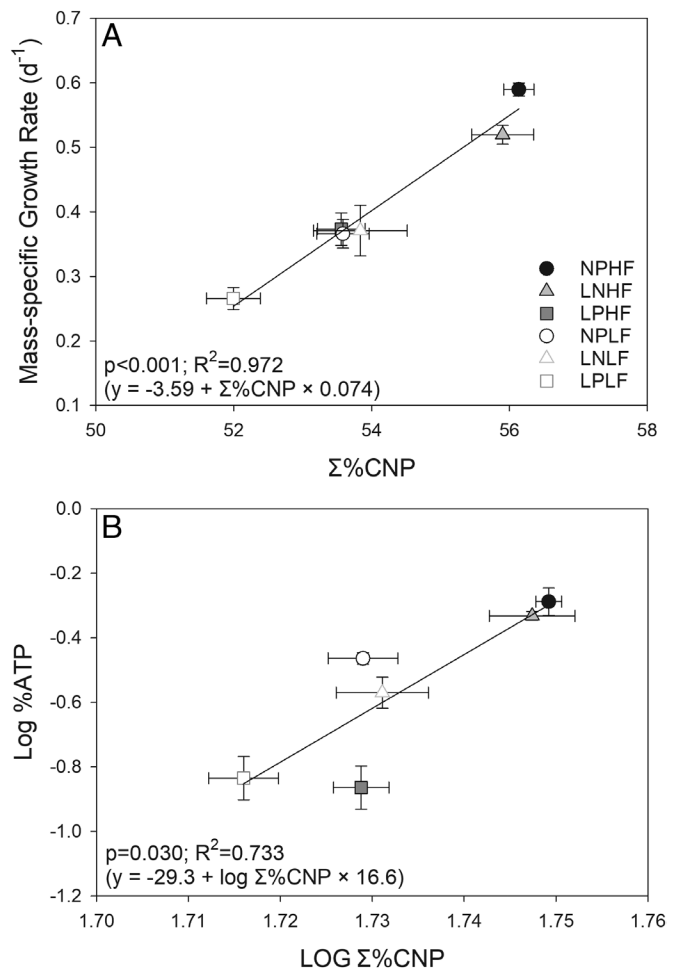


Fig. 2. Stoichiometric growth rate, elemental, and ATP relationships. (A) Relationship between the sum of %C, %N, and %P ($\Sigma\%CNP$) and mass-specific growth rate; (B) log-log relationship between the sum of $\Sigma\%CNP$ and %ATP (note that the ATP axis is inverted to reflect the positive relationship between resources). Means and 95% CIs are shown for each treatment.

and %ATP relationship was maintained across most diet treatments, suggesting homeostatic regulation of this relationship (12, 38, 39). However, animals fed low quantities of high-quality food or high quantities of P-limited food showed altered ATP content relative to their body stoichiometry indicating that this relationship was not fixed. These patterns suggest that the metabolic adjustments responsible for maintaining proportional relationships between *Daphnia* stoichiometry and ATP content along with deviations from this trend are rooted in the differential use efficiencies of these resources in support of the core processes of biomass production, the synthesis and use of ribosomes and proteins.

Relative use efficiency imbalances can help explain unique organismal metabolic adjustments to different forms of resource limitation (Fig. 3). *Daphnia* fed high quantities of both low-N and low-P food showed classic patterns of higher use efficiencies for limiting resources in these treatments (40). N-limited animals achieved near-maximal growth rates by balancing their N and ATP use efficiencies, with only slight declines in use efficiencies of C and P. Due to the reduced N content of N-limited *Daphnia*, their high growth rates cannot be explained by protein production alone and are also likely related to the accumulation of C-rich lipid stores as demonstrated previously (41). Lower P-use efficiency in these animals is related to the accumulation of excess P under N-limitation, a response that is known to “break” relationships between growth and P content predicted by the growth rate

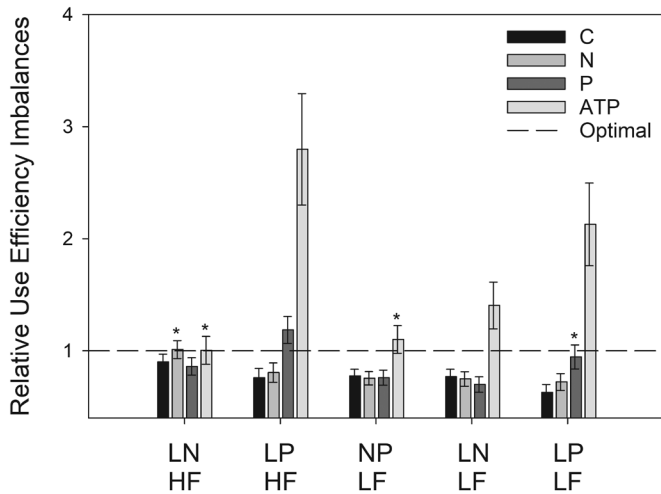


Fig. 3. Relative use efficiency imbalances. Imbalances reflect proportional differences in the use efficiency ratios of organisms in resource-limited environments relative to those of optimally growing NPHF animals. Balanced use efficiencies are denoted (*) where 95% CIs overlap 1.

hypothesis (17). P-limited daphnids more efficiently used P and ATP compared to optimally growing animals; however, this efficiency appeared to come at the cost of reduced C and N use efficiencies in our study. The putative tradeoff between P/ATP and C use efficiencies can be attributed to the decoupling of cellular respiration and ATP production resulting from overflow metabolism under P-limitation (42, 43). Dual limitation by P and readily available energy in the form of ATP could account for the reduced growth rates of P-limited animals compared to those in other high food quantity treatments and explain why they had lower %ATP relative to their $\Sigma\%CNP$ content (Fig. 2B). Reduced N use efficiencies of P-limited daphnids may reflect altered protein synthesis rates resulting in the accumulation of free amino acids in the cytoplasm as previously reported (44).

Our data also shed light on how stoichiometric food quality interacts with food quantity, as *Daphnia* facing identical stoichiometric ratios in their diets showed differing relative use efficiency responses under low food rations. Animals in all low-food treatments showed higher ATP compared to elemental use efficiencies (Fig. 3), which would be expected under energy limitation. However, animals fed high-quality food showed balanced ATP use efficiencies, suggesting that they were also limited by lower intake and assimilation of elements, resulting in higher %ATP relative to $\Sigma\%CNP$ content in their tissues compared to other treatments (Fig. 2B). N-limited daphnids were unable to balance their N-use for growth and instead appeared to minimize all efficiency imbalances to a similar degree in order to maintain proportional relationships between their elemental and ATP contents. P-limited animals were also able to regulate their $\Sigma\%CNP$ and %ATP relationships (Fig. 2B) and balance their P-use for growth (Fig. 3), but this response corresponded with a greatly reduced C use efficiency. Together, these results indicate that, regardless of the specific form of limitation, relative differences in organismal C, N, P, and ATP contents reflect metabolic adjustments necessary for sustaining efficient biomass production (12, 26, 45).

Despite their metabolic complexities, the physiological adjustments indicated by our data ultimately yielded systematic differences in daphnid ATP and elemental contents that can be used to accurately predict organismal growth rates using absolute use efficiency imbalances. ATP use efficiencies were less imbalanced than those for elements across all treatments (Fig. 4A), underscoring

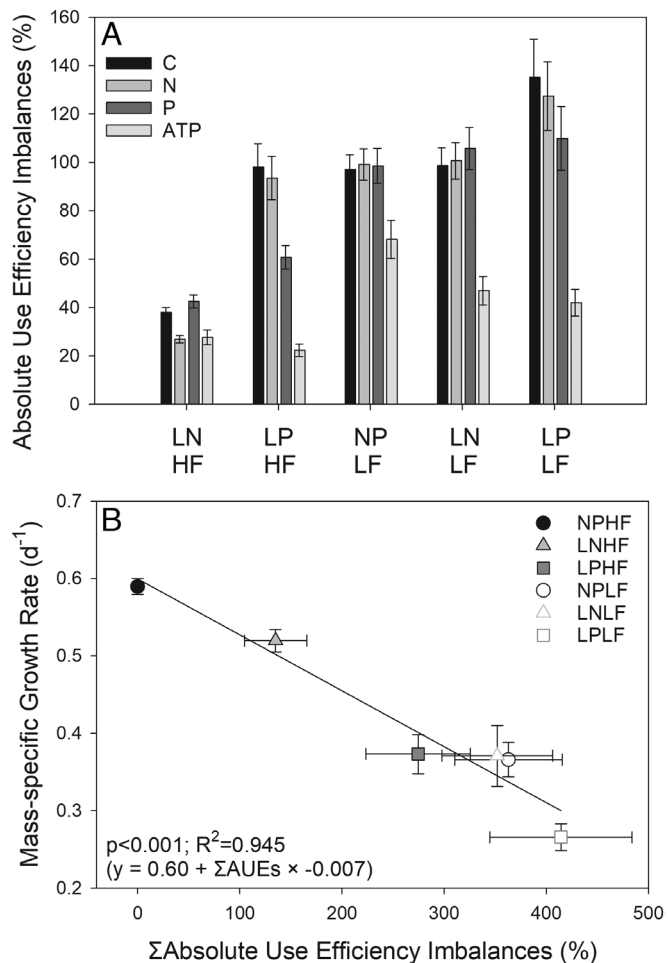


Fig. 4. Absolute use efficiency imbalances. (A) Imbalances reflect %differences in growth efficiencies of organisms in resource-limited environments relative to those of optimally growing NPHF animals. All growth efficiencies are imbalanced relative to NPHF animals, which have use efficiency imbalances of 0. (B) The relationship between total use efficiency imbalances and mass-specific growth rate.

the importance of homeostatic ATP regulation in the cytoplasm (22, 24). However, ATP levels were still lowest in P-limited treatments (Fig. 1B) and animals in these treatments had greater total use efficiency imbalances and lower growth rates than those fed similar quantities of other diets (Fig. 4B). Because *Daphnia* are crustaceans, tradeoffs between metabolic and structural uses of P could explain this pattern as molting is an energetically expensive process (i.e., high ATP demand; 46) and carapace P loss can account for between 14 and 35% of their total P budget (47, 48). Counterintuitively, although absolute use efficiency imbalances were lower for P-limited daphnids under high vs. low food supplies (Fig. 4A), elevated molting rates are likely responsible for the greater imbalances in P and ATP relative use efficiencies in high food treatments (Fig. 3). Nevertheless, the summed imbalances of all resources proved to be robust predictors of growth rate (Fig. 4B) without the need for explicitly accounting for these taxon- or diet-specific details in our model.

Altogether, we have demonstrated the growth efficiency hypothesis to be a comprehensive framework quantitatively linking organismal resource composition and growth to the balance of environmental supplies and efficiency of organismal use for biomass production. By focusing on the interconnected relationships among resources, this framework strengthens stoichiometric theory by further integrating thermodynamic concepts and defining

organismal resource demands in terms of their multivariate requirements for maximum growth. The power of our approach is not an artifact of autocorrelation of body mass and growth rate but is due to the predictable stoichiometric changes in allocation of resources to biochemical production caused by experimental diet manipulations (Fig. 2A and *SI Appendix, Text S2*). While we focused on more commonly studied resources here, our models can theoretically be extended to study limitation by any other element or even more complex macromolecules (*SI Appendix, Text S3*). We do not claim that our specific model formulations will generalize to all resource environments and organisms, as this would require exhaustive further testing. However, by focusing on the systems-level connections among metabolic pathways common to all organisms (3, 16), similar multivariate imbalance models should be able to predict growth responses to resource limitation more widely (37). Use efficiency imbalances are derived solely from the measurement of organismal resource contents, meaning that they should remain coupled even in more heterogeneous natural environments. Thus, the growth efficiency hypothesis should be useful for assessing the occurrence, severity, and effects of multivariate resource limitation on organismal growth under natural conditions as well as on the integrated flows of energy and elements in ecosystems.

Materials and Methods

Experimental animals were derived from a clonal line of *D. pulex* (49), which was initiated from a single parthenogenetically reproducing neonate and propagated through three generations to generate large numbers of genetically similar animals. *Daphnia* broods (15 animals each) were raised in synthetic N- and P-free COMBO media (50) and fed ad libitum concentrations of the freshwater chlorophyte *Chlamydomonas reinhardtii* (CC-1690; Chlamydomonas Resource Center) that was grown in COMBO media under high light conditions ($\sim 150 \mu\text{mol m}^{-2} \text{s}^{-1}$) in continuous chemostats. These algal cultures were supplied with theoretically balanced supplies of dissolved N and P (N:P 16:1; 13) and diluted at near maximum rates (2.5 d^{-1}). This high-quality food was also fed to animals in NP treatments, whereas N- and P-limited algae for experiments was produced by reducing flow rates and altering N and P supplies in the media (LN: 0.12 d^{-1} , N:P = 0.25; LP: 0.08 d^{-1} , N:P = 150). Algal quantities and C:N ratios were determined by filtering algae onto precombusted and weighed $0.2 \mu\text{m}$ ashed glass fiber filters, drying and weighing samples, and measuring their C and N content using an Elementar MICRO cube elemental analyzer. Algae P samples were filtered onto cellulose acetate filters and analyzed using

inductively coupled plasma optical emission spectroscopy (Thermo Scientific, iCAP 7400).

For growth experiments, neonates (<18 h old) were pooled from brood jars and randomly placed in separate experimental treatment jars containing 15 animals each. Animals from a subset of these jars ($n = 5$) were immediately saved for initial mass measurements using a microbalance ($\pm 0.1 \mu\text{g}$) and the rest were randomly assigned to diet treatments. Algal C:N:P ratios of *Daphnia* experimental diets were NP 124:16:1; LN 152:2:1; LP 755:99:1. Experimental animals were fed daily, with those in high food treatments receiving 2 mg C L^{-1} and those in low food treatments were fed 0.25 mg C L^{-1} . Growth experiments lasted for 5 d, after which animals from separate replicate jars were saved for final mass, C, N, P, and ATP measurements (μg). *Daphnia* for mass and elemental measurements were triple rinsed, put into separate aluminum tins, dried, weighed, and CN and P measurements were made using analytical methods outlined above. For ATP measurements, the mass of individual *Daphnia* was estimated from length-mass regressions developed for this clone for each experimental food treatment. The length was measured to the nearest $1 \mu\text{m}$, and individual *Daphnia* were triple-rinsed and transferred to a sterile ATPase free 0.6 mL microcentrifuge tube. Cold methanol ($-20 \text{ }^\circ\text{C}$) was immediately added to these tubes, and *Daphnia* were mechanically homogenized using a motorized pestle. Homogenate ATP-content was then measured, using a luminescence assay (CellTiter-Glo; Promega) according to ref. 42.

Elemental, ATP, body mass, and mass-specific growth rate data were used to calculate use efficiency ratios using equations derived above. Uncertainties associated with each measurement were propagated in quadrature (i.e., calculating the square root of the sum of squares) across each step of these calculations (10) and used to calculate 95% CIs. All calculations were done using Microsoft Excel (version 16.79.2).

Data, Materials, and Software Availability. Data are available online at <https://doi.org/10.6084/m9.figshare.25595922.v1> (51). No code was used in this analysis.

ACKNOWLEDGMENTS. We thank P.C. Frost, N.D. Wagner, and A.L. Peace for constructive feedback that greatly improved the clarity and quality of this manuscript. We also thank B. Nelson and A. Sutton for laboratory culturing assistance and S. Beaupre and M. Sundaram for statistical guidance. This work was supported by the US NSF Rules of Life program (DEB-1930816 to J.J.E. and DEB-1930736 to P.D.J.).

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