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RESEARCH ARTICLE

Stable isotopes reveal sex- and context-dependent amino acid routing in green anole lizards (*Anolis carolinensis*)

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ABSTRACT

Allocation of acquired resources to phenotypic traits is affected by resource availability and current selective context. While differential investment in traits is well documented, the mechanisms driving investment at lower levels of biological organization, which are not directly related to fitness, remain poorly understood. We supplemented adult male and female *Anolis carolinensis* lizards with an isotopically labelled essential amino acid (^{13}C-leucine) to track routing in four tissues (muscle, liver, gonads and spleen) under different combinations of resource availability (high- and low-calorie diets) and exercise training (sprint training and endurance capacity). We predicted sprint training should drive routing to muscle, and endurance training to liver and spleen, and that investment in gonads should be of lower priority in each of the cases of energetic stress. We found complex interactions between training regime, diet and tissue type in females, and between tissue type and training, and tissue type and diet in males, suggesting that males and females adjust their ^{13}C-leucine routing strategies differently in response to similar environmental challenges. Importantly, our data show evidence of increased ^{13}C-leucine routing in training contexts not to muscle as we expected, but to the spleen, which turns over blood cells, and to the liver, which supports metabolism under differing energetic scenarios. Our results reveal the context-specific nature of long-term trade-offs associated with increased chronic activity. They also illustrate the importance of considering the costs of locomotion in studies of life-history strategies.

KEY WORDS: Activity, Allocation, Life history, Locomotion, Performance, Reptile, Trade-off

INTRODUCTION

A fundamental premise of life-history theory is that acquired energetic resources are allocated to different aspects of the integrated organismal phenotype depending on the current ecological, selective and energetic context (Van Noordwijk and Dejong, 1986; Stearns, 1989). Because these contexts are dynamic, organisms are expected to adjust their allocation strategies when possible throughout their lifetimes to maximize residual reproductive value (Kokko, 1998; Royle et al., 2006). Although such strategies are understood in general terms at levels of biological organization that are most proximate to fitness (i.e. the classic trade-off between reproduction and survival), the mechanisms driving these trade-offs at levels below that of the whole organism have received far less attention (Zera and Harshman, 2001; Husak and Lailvaux, 2022). While many of the mechanisms that modulate life-history trade-offs are conserved across metazoan animals, they operate in context-specific ways and could impact phenotypic expression and trade-offs which may not be immediately apparent, depending on the type and extent of environmental variation in question.

Energy limitation accompanied by increased energy demand can result in significant trade-offs; but because there is no one-size-fits-all response to environmental variation, the nature of that energetic demand can drive distinct patterns of trade-offs that might also vary depending on the extent of energetic constraint. For example, significantly increased activity may suppress aspects of the immune system (Pedersen and Hoffman-Goetz, 2000; Wang and Husak, 2020), whereas decreased caloric intake may decrease growth (Le Galliard et al., 2005; Lailvaux et al., 2012); however, the combination of the two could result in an entirely different response, such as decreased reproductive investment (Husak et al., 2016). In addition to these context-dependent effects on traits, the trade-offs invoked in response to specific environmental challenges might also be sex specific, with males and females prioritizing different fitness-enhancing traits and thus manifesting different patterns of energetic investment (Cox et al., 2008; Maklakov et al., 2009). Understanding the different mechanisms underlying these disparate trade-offs is important if we are to predict life-history responses to specific environmental challenges.

Studies of phenotypic trade-offs, both correlative and experimental, have been useful for understanding how the integrated organismal phenotype can dynamically deal with environmental challenges (Ghalambor et al., 2003; Ghalambor et al., 2004), but those studies do not provide direct evidence that the traits of interest 'compete' for macromolecules (McCue, 2011). Stable isotopes provide a useful way to test whether different phenotypic traits draw from the same resource pool, ruling out shared mechanistic pathways as the sole cause of trade-offs (Husak and Lailvaux, 2022). Isotopically labelled molecules, such as amino acids or fatty acids, can be given as supplements to individuals during experimental manipulations to determine the fate of those labelled molecules. While this technique has been used extensively to study the tissue-specific fates of nutrients in humans during and after exercise training (Wilkinson et al., 2017), it remains less common in studies of other animals (O'Brien et al., 2008; Pettit et al., 2019). Larval female *Drosophila melanogaster* fed a reduced diet of isotopically labelled yeast (^{13}C and ^{15}N) revealed a higher ratio of somatic tissue to egg investment of carbon, nitrogen and essential amino acids compared with females on a full diet (O'Brien et al., 2008). Tracking a ^{13}C-enriched sugar solution fed to ruby-throated hummingbirds (*Archilochus colubris*) before sleeping showed that a large portion of the evening meal was converted to fats, which were then later catabolized to support overnight metabolism, sparing existing energy stores (Eberts et al., 2019). Studies on lizards have also revealed direct evidence of shared resource pools for

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competing phenotypic demands. For example, male brown anole lizards (*Anolis sagrei*) increased allocation of isotopically labeled amino acids (^{13}C-leucine) to their livers with increasing doses of injected bacterial lipopolysaccharide (LPS), revealing a cost of immune activation (Brace et al., 2015). Pettit et al. (2019) used exogenous ^{15}N-leucine and ^{13}C-1-palmitic acid to determine the fate of lipids and amino acids in eggs and to healing wounds of side-blotched lizards (*Uta stansburiana*). Durso and French (2017) similarly used ^{15}N-leucine to show that female side-blotched lizards allocated high amounts of amino acids to eggs or wounds, but not both, providing clear evidence of a trade-off between reproduction and immunity. These studies make it clear that stable isotopes hold a great deal of potential for understanding the targets and extent of resource allocation in contexts besides immune function.

Locomotor performance is a common target of selection and affects fitness in ecological contexts ranging from foraging and predator escape to male combat and dispersal (reviewed in Husak and Fox, 2008; Irschick et al., 2008; Husak, 2016). The type of locomotor performance used varies both within and among species, and these different locomotor modes exhibit distinct patterns of trade-offs both with each other (Pasi and Carrier, 2003; Careau and Wilson, 2017a,b) and with other life-history traits (reviewed in Lailvaux and Husak, 2014). Endurance and sprint performance are further distinguished interspecifically by their differences in susceptibility to allocation-based life-history trade-offs. For example, maximum endurance capacity covaries with several life-history traits including longevity, age at maturity, and offspring size across 25 species of phrynosomatid lizards, yet maximum sprint speed is unrelated to any measured life-history traits across these same species (Husak and Lailvaux, 2017). In addition, males and females frequently exhibit differences in both the level of investment in locomotor traits throughout their lifetime (Lailvaux et al., 2011) and their sensitivity to allocation-based trade-offs prompted by locomotor investment (Husak et al., 2021). From a mechanistic perspective, these trade-offs should intuitively be driven by alterations to the physiological systems that power locomotor capacities; however, data that directly speak to those physiological mechanisms whereby allocation- and acquisition-based changes in locomotor performance affect the expression of other aspects of the integrated organismal phenotype, as well as how those mechanisms might differ in males and females, are scarce.

Lizards have long been a model system for the study of locomotion, and over the past decades have also become useful organisms for understanding life-history trade-offs. An emerging literature on green anole (*Anolis carolinensis*) lizards in particular is showing that increasing investment in locomotor capacities via exercise training not only affects those locomotor abilities and related physiological systems (Husak et al., 2015; Lailvaux et al., 2018) but also prompts trade-offs with key fitness-related phenotypes such as immunity (Husak et al., 2017), growth and reproduction (Husak et al., 2016). These studies also show that different kinds of exercise training provoke different trade-offs, with sprint and endurance training having distinct effects on various aspects of innate immune function, whereas acquired immunity is unaffected by any kind of training (Wang and Husak, 2020). Furthermore, trade-offs may be sex specific (Lailvaux et al., 2019; Husak et al., 2021). Although some studies have considered how exercise training apportions resources to specific organs through measurement of gross indicators such as heart ventricle mass (Husak et al., 2016), we currently lack an understanding of the general allocation patterns to such organs under different training and resource availability contexts.

In this study, we used a combination of exercise training and dietary manipulation to measure patterns of amino acid routing under different energetic contexts in adult male and female green anole lizards. Our aim was to test the general hypothesis that different training regimes under varying resource conditions drive allocation to different tissues and organs. We quantified these patterns by administering exogenous doses of ^{13}C-1-L-leucine to both trained and control animals subject to differing resource availability. Leucine is an essential amino acid and common protein component that cannot be synthesized by the body. Tracking the amount of the ^{13}C tracer in target tissues of interest therefore serves as a proxy for protein synthesis in those tissues (McCue et al., 2011). We then measured enriched ^{13}C amounts in skeletal muscle, liver, gonads and spleen. Because of the potentially different trade-offs prompted by endurance and sprint training, respectively, we trained separate groups of males and females for both kinds of locomotion. Our previous work revealed that endurance training increases hematocrit and heart ventricle mass, but also increases growth and decreases bacterial killing ability of plasma (Husak et al., 2015, 2016). Sprint training, in contrast, increases the cross-sectional area of fast-glycolytic muscle fibers in leg muscles (Husak et al., 2015). Reducing calories has some similar, but also some opposing, effects to training. Calorie restriction decreases hematocrit, growth, reproductive investment by both sexes and liver mass, as well as immunocompetence (Husak et al., 2016). These results allowed us to make predictions about where lizards might prioritize routing of ingested amino acids under these energetic challenges. Specifically, we predicted that sprint training would result in high allocation of leucine to skeletal muscle, whereas endurance training would result in high routing to the spleen, as a result of increased red blood cell production and turnover, as well as lymphocyte turnover (Smith, 1995; Nielsen, 2003; Shephard, 2016; Montero et al., 2017; Husak et al., 2021). We further predicted calorie restriction to result in high routing to the liver, for ketogenesis and production of metabolic fuel, but low routing to other tissues. Finally, we predicted low routing to gonads for both sexes and for all treatments compared with controls.

MATERIALS AND METHODS

Husbandry and diet treatments

We acquired 59 adult female and 61 adult male, wild-caught *Anolis carolinensis* Voigt 1832 lizards from a commercial pet dealer (Candy's Quality Reptiles, LaPlace, LA, USA). Lizards were housed in 12 l medium cages (Kritter Keepers, Lee's Aquarium and Pet Products, San Marcos, CA, USA) in male–female pairs. The housing room was maintained at 29–31°C on a 12 h:12 h light:dark cycle, and lizards were acclimated for 2 weeks prior to beginning the experiment (as in Husak et al., 2015, 2016). Each lizard was randomly allocated to one of three training groups (control, sprint training or endurance training). In addition, each lizard was also assigned to one of two diet treatments which we refer to here as either a high-diet treatment of three adult crickets dusted with vitamin powder 3 times per week [equivalent to '*ad libitum*' in Lailvaux et al. (2012) and Husak et al. (2015), and considered here as the control situation], or a reduced-diet treatment of one adult cricket dusted with vitamin powder 3 times per week consistent with previous studies (Lailvaux et al., 2012; Husak et al., 2016; Lailvaux et al., 2020; Marks et al., 2021). Lizards were observed to ensure that each of the pair consumed their crickets. Importantly, we note that the high diet provides a level of nutrition that leads to phenotypic trade-offs when lizards are energetically challenged,

such as through training or other activities (Husak et al., 2016; Wang and Husak, 2020; Husak et al., 2021). Therefore, each of the 12 sex/diet/training combinations was allocated n=10 individuals, with the exception of female/high diet/control (n=9) and male/high diet/control (n=11). We measured mass and snout–vent length (SVL) of each lizard both prior to commencement of training and once again post-training. All procedures were done with approval by the University of St Thomas Animal Care and Use Committee (protocol 80).

Training

Sprint- and endurance-trained lizards were subject to standard training regimes for this species that elicit enhanced performance, physiological responses to training and phenotypic trade-offs (Husak et al., 2015; Husak et al., 2016; Wang and Husak, 2020). The training regimes are meant to reflect the high end of naturally observed activity in the wild, and the ecological relevance of our methods is described in more detail elsewhere (Wang and Husak, 2020). Briefly, endurance-trained lizards were placed on a flat (0 deg incline) treadmill set to 0.18 km h^{-1} for 30 min, 3 times per week for 9 weeks. At weeks 2 and 4, the incline of the treadmill was increased to 9 deg and 13 deg, respectively, to increase training intensity. This protocol has repeatedly been shown to elicit increases in endurance capacity relative to untrained controls in this species (Husak et al., 2016; Sorlin et al., 2022). Sprint-trained lizards were trained 3 days a week for 9 weeks, with each lizard being run 3 times in 1 day (trials separated by 1 h), following Husak and Lailvaux (2019). The racetrack was a 2 m long, 5 cm diameter dowel covered in cork (for traction) and placed at a 45 deg angle to simulate natural conditions (Cox et al., 2009). Intensity was increased every 3 weeks by attaching pipette-tip weights around the lizards' waists with loosely tied string. Mass of the weights was increased by adding putty into the pipette tip. Lizards started with no weights attached, and then progressed to approximately 25% of the average body mass by sex (0.3 g for females, 1 g for males) and ended with 50% of the average body mass (0.6 g for females, 2 g for males).

Isotope treatment and analysis

The day after the last day of training, each individual lizard received an oral dose of 4 mg 99% ^{13}C-1-L-leucine suspended in 40 µl of sunflower oil (Cambridge Isotope Labs, Cambridge, MA, USA) following Brace et al. (2015). Lizards were rapidly decapitated 24 h later and tissue samples collected and kept at −80°C until prepared for isotope analysis. This timeline followed Brace et al. (2015) so that our results would be comparable. We collected skeletal muscle (gastrocnemius), liver, gonad (testis or ovary) and spleen tissues from dissected lizards. Samples were dried at 60°C, ground, and weighed to the nearest 0.001 mg on an ultra-microbalance (Mettler Toledo UMX2). Whole skeletal muscles, gonads and spleens were analyzed because of their small size, whereas the larger livers had samples of approximately 2 mg (mean±s.e.m. 1.81±0.08 mg) taken from ground tissue. Two male individuals that did not receive doses of ^{13}C-leucine were used to measure background tissue values, plus an additional six individuals (3 males and 3 females) for the liver only. Although tissues of lizards vary in their incorporation of carbon (Warne et al., 2010), with the more metabolically active liver being faster than muscle (Murray and Wolf, 2012), our design should still have been timed appropriately to determine differences among treatments in the tissues we examined (Warne et al., 2010). In the absence of existing data on the time course of the exercise response in green anoles, we focused on the resulting phenotypic trade-offs due to chronic increased activity (i.e. 'exercise'), rather than attempting to time ^{13}C-leucine administration for peak sensitivity. Furthermore, carbon incorporation rates increase with decreasing body size, so our smaller species should have carbon incorporation that is faster than that of the previously studied lizard *Sceloporus undulatus* (Warne et al., 2010). Although portions of orally administered ^{14}C-leucine are oxidized to CO_2 or remain in blood plasma after 24 h in Sprague–Dawley rats, the majority of it is deposited in various tissues, primarily skeletal muscle, liver and skin, with very little excreted (Lee et al., 2015). Lee et al. (2015) further showed that the dose administered can impact metabolism of leucine, with a high dose (1000 mg kg^{-1}) showing a greater conversion of leucine to β-hydroxy-β-methylbutyrate (HMB) than a low dose (3 mg kg^{-1}). Nevertheless, HMB has been shown to have anabolic effects on muscle, like leucine (Kornasio et al., 2009; Aversa et al., 2011), so the presence of either of these metabolites in skeletal muscle of our samples could be due to the response to sprint training. Leucine and HMB may also be routed to the liver for further oxidation and entry into the citric acid cycle as we predict above. However, we do note that the dynamics of leucine metabolism in reptiles, which have lower metabolic rates than mammals, is relatively unknown, as is the carbon dynamics of terrestrial ectotherms in general. Despite the potential fates of ^{13}C-leucine that we did not quantify, the tissues we did collect should have sufficient ^{13}C-leucine routed to them to test our predictions. Continuous-flow isotope ratio mass spectrometry was performed at the University of California Davis Stable Isotope Facility. ^{13}C-Leucine values measured in each tissue sample were recorded as $\delta^{13}C_{VPDB}$ and represent the difference between the sample and an industry ^{13}C standard (Slater et al., 2001; Brace et al., 2015). The increase in ^{13}C-leucine from the administered tracer (enrichment values) was calculated as the difference between the background tissue values from the undosed individuals and the levels measured in the tissues of those that received the ^{13}C-leucine.

Statistical analyses

We used the R packages *nlme* (https://CRAN.R-project.org/package=nlme) and *lme4* (Bates et al., 2015) to fit general linear mixed models to our data with the aim of testing the hypothesis that different training regimes under varying resource conditions drive routing to different tissues and organs. Because preliminary analyses yielded uninterpretable four-way interactions between sex, diet, tissue type and training, we fitted separate mixed models for males and females (Lailvaux et al., 2019; as in Husak et al., 2021; see also Sorlin et al., 2022). We used Box–Cox transformations on enrichment values +3 to achieve normality and improve model fit. Both models contained transformed enrichment values (raised to the exponent 0.06 for males; 0.42 for females, as determined by Box–Cox transformations) as the dependent variable; diet, training and tissue type with all possible interactions as fixed effects; lizard body mass and tissue sample mass as covariates; and individual as a random effect (because multiple tissues harvested from the same individual are not independent). We used log-likelihood ratio reduction tests in a maximum likelihood framework to assess the significance of predictors and determine the minimum adequate model for each sex, which we then re-fitted using restricted estimate maximum likelihood (REML) (Crawley, 1993; Silk et al., 2020). We forced both models to retain sample mass; we found no evidence of multicollinearity in models containing both sample mass and body mass. We used the *DHARMa* R package (https://CRAN.R-project.org/package=DHARMa) to assess mixed model fit, and the *emmeans* R package (https://CRAN.R-project.org/package=emmeans) to test for specific contrasts among

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combinations of predictor levels using Tukey tests, and to plot marginal means (i.e. effects of each predictor independent of other predictors in the model). We used R version 4.2.2 for all analyses (http://www.R-project.org/).

RESULTS

The minimum adequate model for *A. carolinensis* males retained an interaction between tissue type and diet, and a separate interaction between tissue type and training (Table 1, Fig. 1). Contrasts indicate that the former results are driven by significant effects of diet on the liver but not on any other tissue, such that reduced-diet males exhibited significantly greater ^{13}C-leucine routing to the liver (P<0.001) compared with males on the high (control) diet. Reduced-diet males also showed significantly more routing to the liver than to the gonads (P<0.001), while this was not the case for males on the high diet (P=0.6856). Contrasts also indicate that the interaction between tissue and training is driven by higher ^{13}C-leucine routing to the liver and spleen. Sprint training elicited the highest ^{13}C-leucine routing to the liver compared with both control (P=0.015) and endurance-trained (P=0.009) lizards, while sprint training also resulted in higher investment in the spleen compared with controls (P=0.0055) but not compared with endurance-trained males (P<0.1154). By contrast, training did not influence routing to gonads or muscle. In addition to sample mass, body mass was retained as an important factor in the male model.

The corresponding model for *A. carolinensis* females retained a three-way interaction between tissue type, diet and training, as well as the fixed effects of body mass and sample mass (Table 2, Fig. 2). There was increased routing to the liver in sprint-trained and endurance-trained lizards compared with controls, but that increase was seen only in reduced-diet lizards compared with high-diet lizards. Specifically, sprint-trained, reduced-diet lizards had the greatest ^{13}C-leucine routing to liver compared with controls (P<0.001). Endurance-trained lizards on the reduced diet also showed significantly elevated ^{13}C-leucine investment in the liver relative to controls (P=0.0114), but not significantly different from sprint-trained females on the same diet (P=0.177). Routing to the spleen also increased in sprint-trained, reduced-diet female lizards relative to controls (P=0.002), but not in sprint-trained females on the high diet (P=0.51). Sprint-trained, restricted-diet female lizards also had increased ^{13}C-leucine routing to gonads compared with untrained controls (for reduced diet: P=0.033), and also compared with endurance-trained lizards on the same diet (P=0.0213), but endurance training did not affect ^{13}C-leucine routing to the gonads compared with untrained controls in either the reduced (P=0.99) or high diets (P=0.38). Similar to males, there was no significant effect of either diet or training on ^{13}C-leucine routing to muscle.

DISCUSSION

Organisms allocate resources acquired from the environment to different components of the integrated phenotype depending on both the amount of resources available and the allocation context. These allocation patterns collectively manifest as trade-offs that characterize life-history strategies, yet the proximate mechanisms underlying those trade-offs below the level of the individual are poorly understood (Zera and Harshman, 2001; Lailvaux and Husak, 2014; Husak and Lailvaux, 2022). We tracked an isotopically labelled essential amino acid to measure routing to several key organs during two different, ecologically relevant types of energetic stress – chronic increased activity and reduced calories – as well as a combination of the two, in male and female adult green anole lizards. These data show some unexpected patterns of amino acid allocation to various tissue types under different kinds of energetic stress.

Table 1. Best-fit mixed model describing variation in transformed ^{13}C-leucine allocation in adult male *Anolis carolinensis*

	d.f.	Coefficient	s.e.
Intercept	166	1.21	0.018
Mass	55	–0.01	0.0038
Sample mass	166	0.0004	0.002
Tissue (Liver)	166	–0.0078	0.007
Tissue (Muscle)	166	–0.074	0.008
Tissue (Spleen)	166	0.047	0.009
Training (Endurance)	55	–0.011	0.009
Training (Sprint)	55	0.007	0.009
Diet (Reduced)	55	–0.006	0.007
Tissue (Liver)×Training (Endurance)	166	0.016	0.009
Tissue (Muscle)×Training (Endurance)	166	0.018	0.009
Tissue (Spleen)×Training (Endurance)	166	0.021	0.009
Tissue (Liver)×Training (Sprint)	166	0.025	0.009
Tissue (Muscle)×Training (Sprint)	166	0.011	0.009
Tissue (Spleen)×Training (Sprint)	166	0.021	0.009
Tissue (Liver)×Diet (Reduced)	166	0.038	0.007
Tissue (Muscle)×Diet (Reduced)	166	0.001	0.007
Tissue (Spleen)×Diet (Reduced)	166	0.012	0.007

Coefficients describe the estimated change in ^{13}C-leucine between the categories named in the table and the baseline categories (not shown). The baseline category for 'Tissue' is 'Gonad'; the baseline category for 'Training' is 'Control'; and the baseline category for 'Diet' is 'High'.

Table 2. Best-fit mixed model describing variation in transformed ^{13}C-leucine allocation in adult female *A. carolinensis*

	d.f.	Coefficient	s.e.
Intercept	154	4.86	0.6
Mass	52	–0.88	0.25
Sample mass	154	–0.29	0.055
Tissue (Liver)	154	0.857	0.27
Tissue (Muscle)	154	–0.37	0.27
Tissue (Spleen)	154	1.53	0.278
Training (Endurance)	52	0.414	0.31
Training (Sprint)	52	0.75	0.31
Diet (Reduced)	52	0.12	0.34
Tissue (Liver)×Training (Endurance)	154	–0.19	0.38
Tissue (Muscle)×Training (Endurance)	154	–0.27	0.38
Tissue (Spleen)×Training (Endurance)	154	–0.05	0.38
Tissue (Liver)×Training (Sprint)	154	–0.67	0.38
Tissue (Muscle)×Training (Sprint)	154	–0.31	0.38
Tissue (Spleen)×Training (Sprint)	154	–0.4	0.38
Tissue (Liver)×Diet (Reduced)	154	–0.36	0.39
Tissue (Muscle)×Diet (Reduced)	154	–0.17	0.39
Tissue (Spleen)×Diet (Reduced)	154	–0.65	0.39
Training (Endurance)×Diet (Reduced)	52	–0.43	0.45
Training (Sprint)×Diet (Reduced)	52	0.09	045
Tissue (Liver)×Training (Endurance)×Diet (Reduced)	154	1.168	0.54
Tissue (Muscle)×Training (Endurance)×Diet (Reduced)	154	0.199	0.54
Tissue (Spleen)×Training (Endurance)×Diet (Reduced)	154	0.91	0.55
Tissue (Liver)×Training (Sprint)×Diet (Reduced)	154	1.35	0.54
Tissue (Muscle)×Training (Sprint)×Diet (Reduced)	154	–0.46	0.54
Tissue (Spleen)×Training (Sprint)×Diet (Reduced)	154	0.72	0.55

Coefficients describe the estimated change in ^{13}C-leucine between the categories named in the table and the baseline categories (not shown). The baseline category for 'Tissue' is 'Gonad'; the baseline category for 'Training' is 'Control'; and the baseline category for 'Diet' is 'High'.

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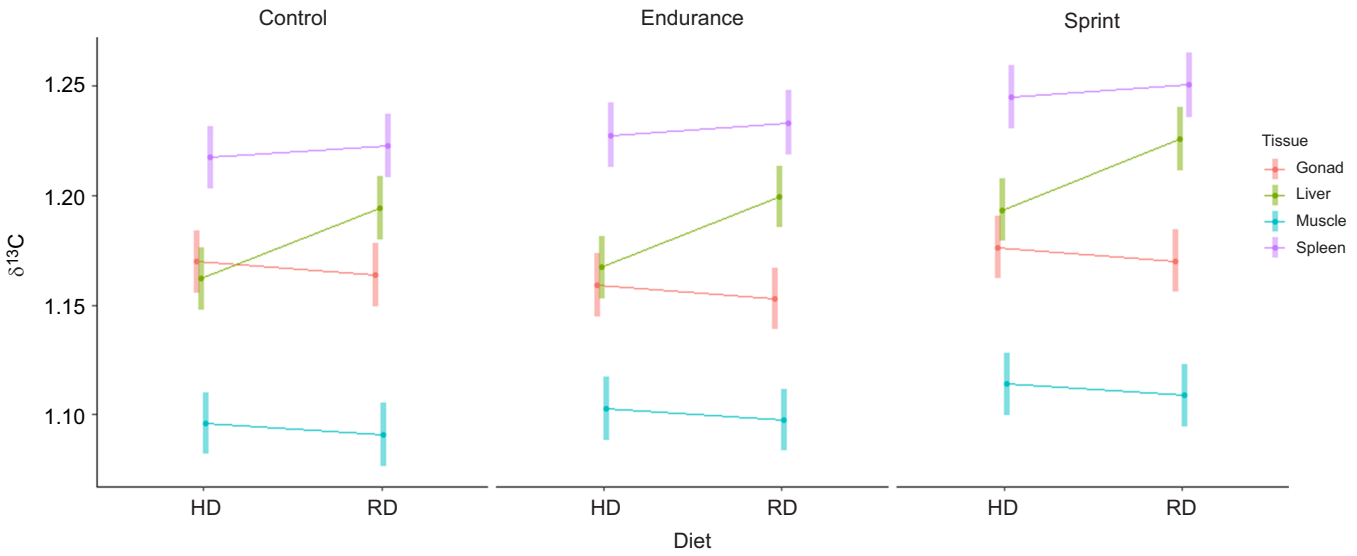

Fig. 1. Male green anole routing of transformed ^{13}C-leucine to four different tissues under different combinations of training and diet regimes. Lizards (*n*=61 total) were allocated to different training (control, no training; endurance and sprint) and diet (HD, high diet; RD, low diet) regimes as indicated. The increase in ^{13}C-leucine (δ^{13}C) measured in the tissues is shown as estimated marginal means ±95% confidence intervals. The model retained separate interactions between tissue type and training, and between tissue type and diet.

Our data indicate that amino acid routing in four green anole tissues is affected by combinations of diet and training. However, we also found distinct patterns of routing in males and females. Within females, routing to tissues depended on the specific combination of diet and training regime (Table 2), whereas in males we found separate interactions between tissue type and diet and between tissue type and training, but no evidence for any interaction between training type and diet (Table 1). A common pattern apparent across both sexes and all treatment combinations is that highest amino acid allocation was observed in the spleen in almost all cases, with the liver appearing to be second most affected. Restricting calories affected routing to the liver in the trained groups differently between males and females, with males apportioning more ^{13}C-leucine to the liver under caloric restriction in all training contexts compared with females. Interestingly, none of the groups had high allocation to skeletal muscle, even those that were sprint trained.

One of our most straightforward predictions was that sprint-trained lizards would route the most ^{13}C-leucine to skeletal muscle. Red blood cells and liver have higher incorporation rates than some other tissues in lizards, but our timing should have allowed detection of routing to muscle, as well as any differences among treatments (Warne et al., 2010). For example, 24 h after oral administration of ^{14}C-leucince in Sprague–Dawley rats, the tissue with the highest amount of resultant ^{14}C-leucine was skeletal muscle (Lee et al., 2015). Previous studies in green anoles show that sprint training of the duration we used increases both cross-sectional area of muscle fibers in leg muscles (Husak et al., 2015) and hepatic expression of insulin-like growth factor (IGF) I and II, albeit the latter in a size-dependent manner (Marks et al., 2022). Contrary to this prediction, however, our data show no evidence of elevated ^{13}C-leucine routing to sampled skeletal muscle in any context. In fact, routing to muscle was lower in some treatment contexts than was routing to gonads, which we had predicted to be lowest of all. The most likely explanation is that most of the increased muscle allocation was already completed at the time of our sampling, analogous to human bodybuilders who plateau in building muscle after some time of training (Wernbom et al., 2007; Damas et al., 2015; Counts et al., 2017). The complex effects of sprint training on IGF expression may also be due in part to the timing of tissue sampling; as in our study, Marks et al. (2022) examined IGF expression after several weeks of training. We would expect that samples taken earlier in the training process would shine important light on the timing of muscle hypertrophy, with ^{13}C-leucine routing to skeletal muscle likely increasing for sprint-trained lizards compared with other groups, and perhaps more straightforward patterns of IGF expression.

A second prediction was that calorie restriction would result in high routing of amino acids to the liver for oxidation to metabolic fuel. Although this prediction was supported, that routing depended on the specific training context. In males, reduced calories resulted in generally increased routing to the liver, with sprint training boosting that routing even further (Fig. 1). In females, diet also impacted the magnitude of ^{13}C-leucine liver routing due to training, with the highest routing again seen in sprint-trained, reduced-diet females (Fig. 2). These results, taken together, suggest that both increased activity and reduced calories result in more breakdown of amino acids for metabolic fuel, but that the type of activity determines the extent to which this occurs. Although leucine is not glucogenic, it is ketogenic such that eventual conversion to acetyl CoA allows entry into the citric acid cycle (Noda and Ichihara, 1976). The routing of exogenous leucine may be part of a larger system of amino acid sequestration and oxidation in the liver, such as conversion to HMB or other metabolites (Lee et al., 2015). Lactate is known to be a source of metabolic fuel via gluconeogenesis in lizards (Gleeson, 1985), but the role of ketogenesis is poorly studied (do Nasciamento et al., 2016). Future studies will help to determine the details in lizards.

Arguably our most striking (and unexpected) finding here is that increased energetic demands from training and restricted calories did not result in lower routing of ^{13}C-leucine to any of the tissues we sampled relative to controls. That is, our experimental lizards routed at least as much exogenous ^{13}C-leucine to the four measured tissues as our control lizards; and certain diet/treatment combinations drove significantly more routing to tissues than the control context. One simple explanation for this pattern is that, without increased leucine

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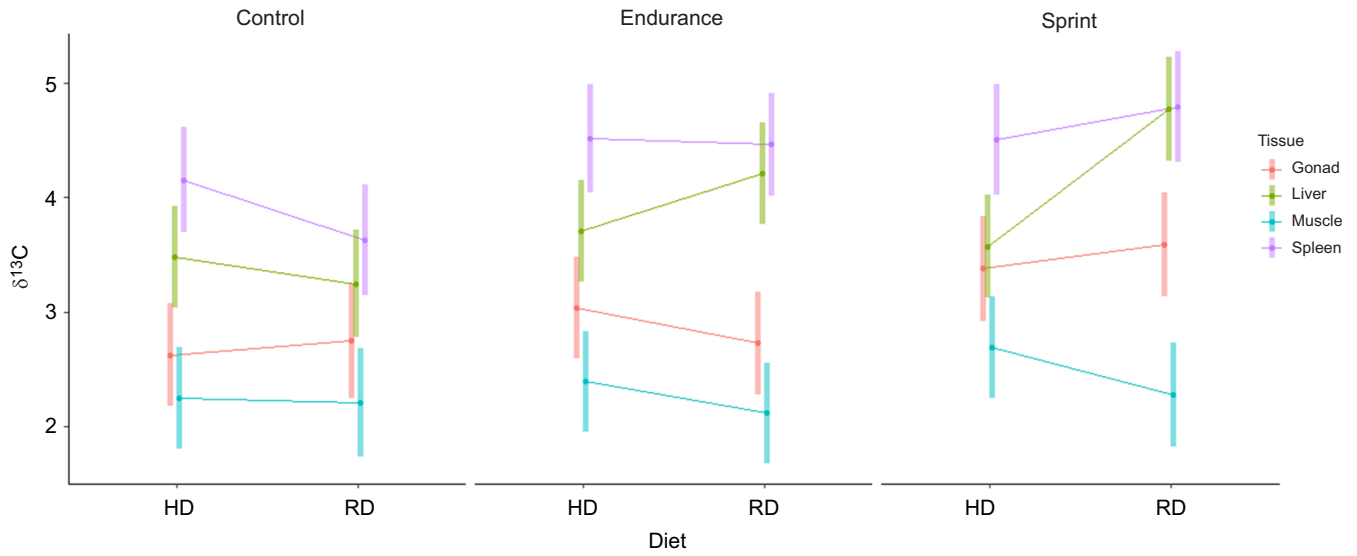

Fig. 2. Female green anole routing of transformed ^{13}C-leucine to four different tissues under different combinations of training and diet regimes. Lizards (*n*=59 total) were allocated to different training (control, no training; endurance and sprint) and diet (HD, high diet; RD, low diet) regimes as indicated, after accounting for effects of tissue mass. Estimated marginal means ±95% confidence intervals are shown. The model retained a three-way interaction between tissue type, training and diet.

demand, control lizards simply excreted the excess amino acid. Another explanation is that control lizards routed the ^{13}C-leucine to tissues that we did not sample, perhaps storing it for use in future reproduction (Warner et al., 2008, 2012). In fact, a restricted diet reduces fat body mass compared with controls in this species (Husak et al., 2016), so sampling fat bodies in future studies would be illuminating. Alternatively, our previous work showed that both endurance and sprint training decreased standard metabolic rate (Lailvaux et al., 2018) due to mitochondrial changes (Reardon et al., 2023). Thus, training actually reduces average energetic expenditure during inactivity, which likely frees up acquired resources, including amino acids, for prioritized traits, including the tissues that we sampled here. A second unexpected finding was that routing to gonads was not lower in trained or calorie-restricted groups compared with controls. Indeed, the sprint-trained, diet-restricted females had higher routing to gonads than controls (Fig. 2). This result is surprising given the general life-history pattern of organisms prioritizing investment in either traits related to reproduction or traits related to survival, but not both (Reznick et al., 1990; Swanson and Dantzer, 2014). Our data show that investment in a survival-related trait (sprinting) might increase investment to gonads as well in certain sexes and contexts, perhaps as a result of changes in IGF expression. However, Husak and Lailvaux (2019) found that sprint training actually reduces survival in an experimental population of green anoles. These results suggest that the relationships among reproductive traits and traits ostensibly related to survival might be more complex than generally appreciated, particularly under different conditions that restrict resource acquisition or different forced allocation regimes (see also Husak et al., 2006, 2008, for experimental evidence linking sprint speed to reproduction in lizards).

Our prediction that endurance training would result in high routing to the spleen, due to increased blood cell turnover (reviewed in Stewart and McKenzie, 2002), was supported by our results, but our data show that sprint training resulted in high routing to the spleen as well. In both types of training, reduced calories further increased routing to the spleen. Calorie-restricted mice showed delayed maturation of natural killer cells and T cells in the spleen, as well as elevated interleukin-7 (IL-7) production, suggesting an increase in lymphocyte cell production and turnover (White et al., 2017; see also Yan et al., 2021; Asami et al., 2022). These dynamics have not been studied in lizards to our knowledge, but offer a fruitful avenue of future research (see Mondal and Rai, 1999, 2001, 2002 for an *in vitro* perspective). Our previous work showed that endurance training increases hematocrit (Husak et al., 2015; 2016), as also seen in human athletes (Shephard, 2016). The different effects of calorie restriction and exercise on blood cell dynamics support our results, where both exercise and calorie restriction separately resulted in higher ^{13}C-leucine to the spleen compared with controls, and the combination resulted in even higher routing. Although these results seem contradictory with our previous work showing lower immunocompetence in endurance-trained lizards (Husak, Ferguson and Lovern, 2016), they are not. Our previous measure of immunocompetence was bacterial killing ability of plasma, a measure of the complement system (McQuillen et al., 1994). The documented effects of calorie restriction and exercise on immune function largely center on T cells and leukocytes (Shephard, 2016), which would not be reflected in a bacterial killing assay. Thus, the results of our current study add a layer of complexity to our understanding of how lizards facing increased energetic demands modify their immune systems.

In conclusion, our data provide evidence for sex- and context-specific investment in various tissues in green anoles trained for different locomotor performance capacities under differing resource availability. Crucially, these results show that ^{13}C-leucine is routed not to those tissues that are most proximate to performance, such as muscle, but to other tissue types that either support metabolism or are involved in other aspects of the whole-organism phenotype that might be affected by our treatments. This study highlights the complexity of resource allocation to traits such as performance that are bolstered by several aspects of metabolism and intimately involved with other components of the integrated organismal phenotype. Future studies of phenotypic trade-offs will greatly benefit from considering the production and maintenance costs of dynamic, whole-organism performance traits directly (Husak and Lailvaux, 2017), as well their morphological and physiological

underpinnings (Lailvaux and Husak, 2014), each of which can complicate patterns of allocation under different types of energy limitation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.F.H., S.P.L.; Methodology: J.F.H., S.P.L.; Validation: J.F.H., S.P.L.; Formal analysis: J.F.H., S.P.L.; Investigation: J.F.H., S.P.L.; Resources: J.F.H.; Data curation: S.P.L.; Writing - original draft: J.F.H., S.P.L.; Writing - review & editing: J.F.H., S.P.L.; Visualization: S.P.L.; Supervision: J.F.H.; Project administration: J.F.H.

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Data availability
The data that support the findings of this study are openly available in the figshare repository: https://figshare.com/s/9a0476b96948c29adc5d. The repository has four files: the main dataset of all data used in a CVS file, isotope_ms_R; a text document with details, README; the R code used to conduct the analysis, Ac isotope ms; and an HTML file with a walkthrough of the analyses, Ac-isotope-ms. For additional information, please contact the authors.

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