Macronutrient balance, reproductive function, and lifespan in aging mice

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In invertebrates, reproductive output and lifespan are profoundly impacted by dietary macronutrient balance, with these traits achieving their maxima on different diet compositions, giving the appearance of a resource-based tradeoff between reproduction and longevity. For the first time in a mammal, to our knowledge, we evaluate the effects of dietary protein (P), carbohydrate (C), fat (F), and energy (E) on lifespan and reproductive function in aging male and female mice. We show that, as in invertebrates, the balance of macronutrients has marked and largely opposing effects on reproductive and longevity outcomes. Mice were provided ad libitum access to one of 25 diets differing in P, C, F, and E content, with reproductive outcomes assessed at 15 months. An optimal balance of macronutrients exists for reproductive function, which, for most measures, differs from the diets that optimize lifespan, and this response differs with sex. Maximal longevity was achieved on diets containing a P:C ratio of 1:13 in males and 1:11 for females. Diets that optimized testes mass and epididymal sperm counts (indicators of gamete production) contained a higher P:C ratio (1:1) than those that maximized lifespan. In females, uterine mass (an indicator of estrogenic activity) was also greatest on high P:C diets (1:1) whereas ovarian follicle number was greatest on P:C 3:1 associated with high-F intakes. By contrast, estrous cycling was more likely in mice on lower P:C (1:8), and the number of corpora lutea, indicative of recent ovulations, was greatest on P:C similar to those supporting greatest longevity (1:11).

aging | macronutrients | lifespan | reproduction | nutrition

Nutrition profoundly influences reproduction and lifespan. Traditionally, dietary restriction has been the central focus of most research, with numerous studies showing that caloric restriction can improve age-related health and prolong lifespan across a wide range of taxa ranging from yeasts to humans (1–6). The extension of lifespan under caloric restriction has been explained by resource-mediated tradeoffs between reproduction (age of sexual maturity, number of offspring, and parental investment) and longevity (senescence and lifespan), with these outcomes titrated for maximum overall reproductive success under given levels of resource availability (7, 8). However, the view that there is a simple constitutive tradeoff between reproduction and longevity has been challenged by accumulating experimental evidence from invertebrate models, beginning with experiments in *Caenorhabditis elegans*, demonstrating that lifespan in long-lived mutants was not affected by the ablation of germ cell and somatic gonad precursors, indicating that neither germ cells nor progeny production was directly responsible for increased longevity in the worm (9). Later, it was found that manipulating the insulin/insulin-like growth factor-1 (IGF1) pathway in adult worms can extend lifespan without the loss of fecundity (10). In beetles, the increased risk of death associated with high egg producing females was uncoupled by increasing food availability (11) whereas studies in *Drosophila* have shown extension of lifespan independent of reproduction and its presumed allocation costs (12–15). Studies using stable isotopes to measure allocation of ingested carbon and nitrogen to eggs and somatic maintenance unambiguously rejected the allocation tradeoff model in *Drosophila* and invoked the idea of direct costs of reproduction to explain the life-history tradeoff between reproduction and lifespan (16).

More recently, however, both the "constitutive tradeoff" and "direct costs of reproduction" models have been superseded by the idea that macronutrients differentially facilitate survival and reproduction and give rise to life-history tradeoffs that constrain trait evolution (5, 15). Experiments in *Drosophila* have shown that the balance of macronutrients, rather than total calorie intake, is a key nutritional factor that influences lifespan and reproduction, and the appearance of a resource-based tradeoff arises because of the different nutritional requirements of these two biological functions (5, 17–20). Such experiments have shown that intake of total calories per se is not responsible for prolonging life, but, rather, the restriction of protein (P) relative to carbohydrate (C) may be the key under ad libitum feeding conditions (5, 18, 21). When individual female flies were confined to one of 28 diets differing in protein and carbohydrate content, lifespan was maximal in those flies provided diets with a low protein to carbohydrate ratio (P:C) whereas reproductive effort, measured by lifetime egg production and egg-laying rate, was maximal on diets containing higher P:C. When provided with

Significance

A fundamental tenet of life-history theory is that reproduction and longevity trade off against one another. Experiments on invertebrates show that, rather than competing for limiting resources, reproduction and lifespan are optimized on different dietary macronutrient compositions. In mice, studies have yet to establish the relationship between macronutrient balance, reproduction, and lifespan. We evaluated the effects of macronutrients and energy on lifespan and reproductive function. Indicators of reproductive function (uterine mass, ovarian follicle number, testes mass, epididymal sperm counts) were optimized by high protein (P), low carbohydrate (C) diets whereas lifespan was greatest on low P:C diets. Corpora lutea and estrous cycling were higher in females on lower P:C diets. Macronutrient balance has profound and opposing effects on reproduction and longevity.

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the opportunity to compose a diet from pairs of foods containing complementary combinations of P:C, flies selected diets that optimized reproductive effort and consequently lived less long than if they had selected a diet of lower P:C. Similar results were reported in field crickets although differences were apparent in the nutrient intakes that best supported measures of reproductive effort in males (calling behavior) and females (egg production) (17). These studies point to a clear link between macronutrient balance, lifespan, and reproduction; however, whether the same patterns are true for mammals has yet to be determined.

Here, we use the Geometric Framework (GF) (22) to measure the effects of energy and macronutrients (protein, carbohydrate, and fat) on reproductive variables and lifespan in aging male and female mice. We show that, as in invertebrates, reproduction and lifespan are regulated by macronutrient balance in mice and that the macronutrient ratio optimal for reproductive function differs from that which maximizes lifespan.

Results

We evaluated the effects of macronutrient balance on reproductive function in 144 male and female mice at 15 months of age. These mice were part of a larger cohort ($n = 858$), the remainder of which were allowed to continue until the ends of their lives for measurement of lifespan (6). Mice were confined to one of 25 diets differing systematically in energy (E) density as well as ratio of P, C, and fat (F), which allowed us to map landscapes for response variables related to reproductive parameters onto arrays of macronutrient and energy intakes (6) (Table S1). To ensure accurate food and energy intake measurements, a custom-made, two-chamber Perspex insert was placed within each cage (6, 23). Food intake was measured weekly for 6 months and monthly thereafter, correcting for food spillage and water content. To aid interpretation, the data surfaces are presented as 2D nutrient heat maps cut through the response surface at the median of the third nutrient axis. Dietary P:C:F compositions are represented as lines (nutritional rails) radiating from the origin in the 3D nutritional space. Animals can move along their nutritional rail by changing food intake but cannot alter the ratio of macronutrients ingested, which is fixed within a diet treatment. In some figures, we have superimposed a nutritional rail that represents a particular nutrient ratio to clarify the relationship between dietary composition and phenotypic outcomes. All surfaces are fitted using thin-plate splines and interpreted statistically using general additive statistical models (GAMs) as previously described (6), the results of which are provided in Fig. S1 and Tables S2–S5.

We have assessed a variety of female and male reproductive responses to evaluate reproductive function, with a focus on the two main reproductive parameters: gamete and steroid production. Uterine mass (an in vivo bioassay for estrogenic activity,

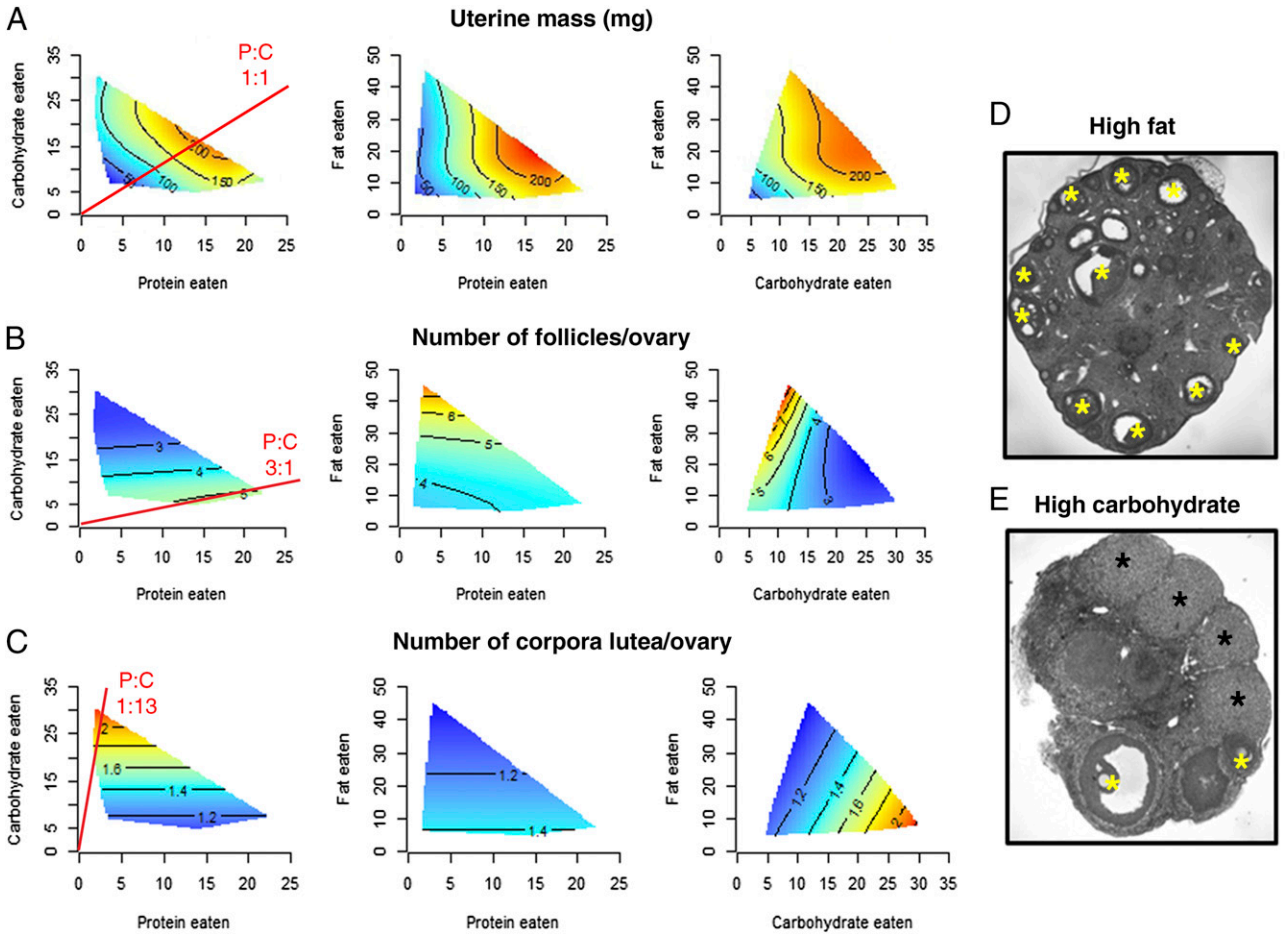

Fig. 1. Macronutrient balance influences female gamete stock. Response surfaces showing the relationship between macronutrient intake (kJ/d) and (*A*) uterine mass, an indicator of steroid production, and (*B*) total number of follicles and (*C*) total number of corpora lutea, both indicators of gametogenesis which were counted across three sections in each ovary. Three 2D slices are presented to show the interactive effects of all three macronutrient dimensions (protein, carbohydrate, and fat). In each 2D slice, the third factor is set at its median. For all response surfaces, areas shown in red indicate the greatest value for each response and fall away as the colors shift from red to blue. The red lines indicate the ratio of macronutrients that maximizes each response. Shown are representative sections of ovaries when (*D*) high fat is consumed and (*E*) high carbohydrate is consumed. Yellow asterisks show follicles, and black asterisks show corpora lutea.

required for triggering ovulation and preparing the uterine microenvironment for embryo implantation and fetal development), average follicle number (reflecting follicular reserve), and the presence of corpora lutea (CL) (reflecting recent ovulations) were evaluated. The amounts and ratios of macronutrients ingested had marked effects on all female reproductive variables reflecting gametogenesis, ovulation, and estrogen secretion. Uterine mass was driven strongly by all three macronutrients (Fig. 1*A* and Table S2), being greatest when P, F, and C intakes were each ~20 kJ/d (i.e., in a 1:1:1 ratio). Follicle number was greatest with high F intake (~45 kJ/d) and in combination with a low P or a low C intake (<10 kJ/d) (Fig. 1 *B* and *D* and Table S2). The number of CL, in contrast, was most affected by C intake (Fig. 1*C* and Table S2), showing maximal numbers when C intake was high (~25–30 kJ/d) and when paired with a low P or low F intake. Unlike other reproductive markers, peak CL numbers occurred on a low P:C ratio of 1:13 (Fig. 1 *C* and *E*). These data show that macronutrients have different effects on measures of female reproductive function. The number of CL and the uterine mass, which are the main parameters reflecting reproductive function, show opposing effects, with a high P:C ratio maximizing uterine mass, but a low P:C ratio maximizing CL number. High-fat intakes, which were most pronounced in mice on low P:F diets as a consequence of compensatory feeding for protein (protein leveraging) (6), were associated with high numbers of follicles and low numbers of corpora lutea, consistent with human data indicating a link between high-fat diets and infertility (24–27).

Estrous cycle stage was determined daily for 11 days by light microscopy of vaginal epithelial cell smears (28). Estrous cycling was maintained in mice as old as 15 months; however, this feature depended upon macronutrient intakes (Fig. 2 and Table S2). High P:C intakes were also associated with increased likelihood of estrous cycling. A dietary P:C ratio of 1:8 (intakes of ~2.5 kJ/d of P and 20 kJ/d of C) was associated with the highest number of cycles across the 11-day observation period, with fat having no statistically significant effect—a result consistent with the effects of fat on CL number: i.e., ovulation (Fig. 1*C*).

To determine the effects of diet on male reproductive function, we measured the mass of the testes and cauda epididymal sperm counts (reflecting gamete production) and the masses of the seminal vesicles and ventral prostate (in vivo bioassays for androgenic activity, required to support spermatogenesis and mating). As expected, both sperm counts and body mass were positively correlated with testes mass ($P = 0.003$ and $P < 0.001$, respectively) (Fig. 3 *A* and *B*). Testes mass was driven strongly by P intake (Fig. 3 *C* and Table S3) even when corrected for body mass (Fig. S1). Smallest testes (~80 mg) were found when mice consumed low amounts of P (~5 kJ/d), and these masses increased steadily to 96 mg as P intake increased to ~20–25 kJ. Testes mass was greatest when the P:C ratio was 1:1. Seminal vesicle and ventral prostate mass were also strongly driven by protein intake (Table S3 and Fig. 3 *D* and *E*). Circulating testosterone levels responded similarly; however, presumably due to the highly pulsatile manner of testosterone secretion (29, 30), these data did not reach statistical significance ($P = 0.098$) (Fig. 3*F*). The results consistently showed that male reproductive function was optimized by high P intake and high dietary P:C.

Lifespan data have been presented previously ($n = 533$) (6) but here are reanalyzed according to sex. The longest median lifespans occurred at low P:C intake ratios (P:C = 1:11 for females; P:C = 1:13 for males) and were significantly influenced by carbohydrate intake ($P = 0.009$ for females; $P = 0.012$ for males) (Fig. 4 and Table S4). Lifespans for males and females showed similar patterns with respect to diet to those reported for the combined cohort (6), with median lifespan greatest on low P:C intake ratios of 1:13. The response surfaces for lifespan differed substantially from those for measures of reproductive function, with this effect being more consistent in males compared with females. To compare these outcomes, the P:C ratios that supported maximal reproductive outcomes were superimposed onto the lifespan surfaces in Fig. 4. Uterine and seminal vesicle mass, both highly steroid-dependent responses, were maximized on substantially higher P:C ratios than for median lifespan (1:1). However, the nutritional optima of corpora lutea number and testes mass, indicators of gamete supply, varied between the sexes. Testes mass was also maximal on higher P:C ratios when compared to diets that maximized lifespan, but CL number, interestingly, was optimized on a similar nutritional rail as lifespan (i.e., low P:C).

Discussion

Recent studies in invertebrates (5, 14, 17, 21, 31) have shown that the balance of macronutrients, not total energy, is a major determinant of reproductive effort and lifespan when food is available ad libitum. Here, we provide evidence that supports these findings for mice and shows that, as in invertebrates, the nutritional optima of reproduction and lifespan differ. These findings highlight the importance of using a robust multidimensional nutritional framework to disentangle the effects of necessarily constrained choices of energy and specific macronutrients (22, 32, 33).

Our data define the macronutritional determinants that maintain reproductive function in aging mice (34). As seen from the response surfaces, for all reproductive parameters measured in both males and females, low total energy intake was associated with decreased reproductive function (blue regions on the surfaces). Such data are consistent with studies on the effects of caloric restriction on fertility, which have generally shown that reduced food intake is associated with impaired fertility (35–40). Of more interest are the impacts of macronutrient balance on reproductive outcomes. In females, macronutrition has differing effects on steroid-dependent responses and indicators of gamete-production potential. Uterine mass was greatest in mice with the highest P:C intakes in the order of 1:1. Estrous cycling was greatest in female mice where P:C ratio was 1:8 whereas numbers of corpora lutea were greatest when on a low P:C ratio of 1:13. In males, the data for steroid-dependent responses and gamete-production potential were more consistent, with the mass of the testes and seminal vesicles greatest in mice with P:C intakes of ~1.1. These data show that the effect of macronutrients on reproductive function differs with sex. To date, a study in field crickets is the only investigation to explore male and female

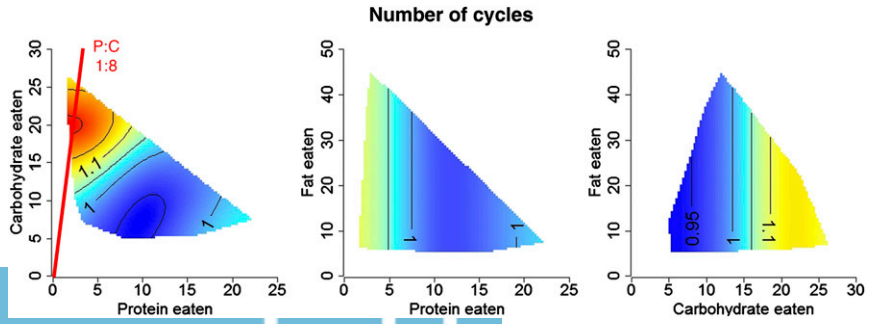

Fig. 2. Estrous cycling is optimized at a high P:C ratio. Response surfaces showing the interactive relationship between macronutrient intake vs. the number of estrous cycles over 11 days. For response surfaces, the third dimension is sliced at the median. Areas shown in red indicate the greatest value for the average number of cycles and fall away as the colors shift from red to blue.

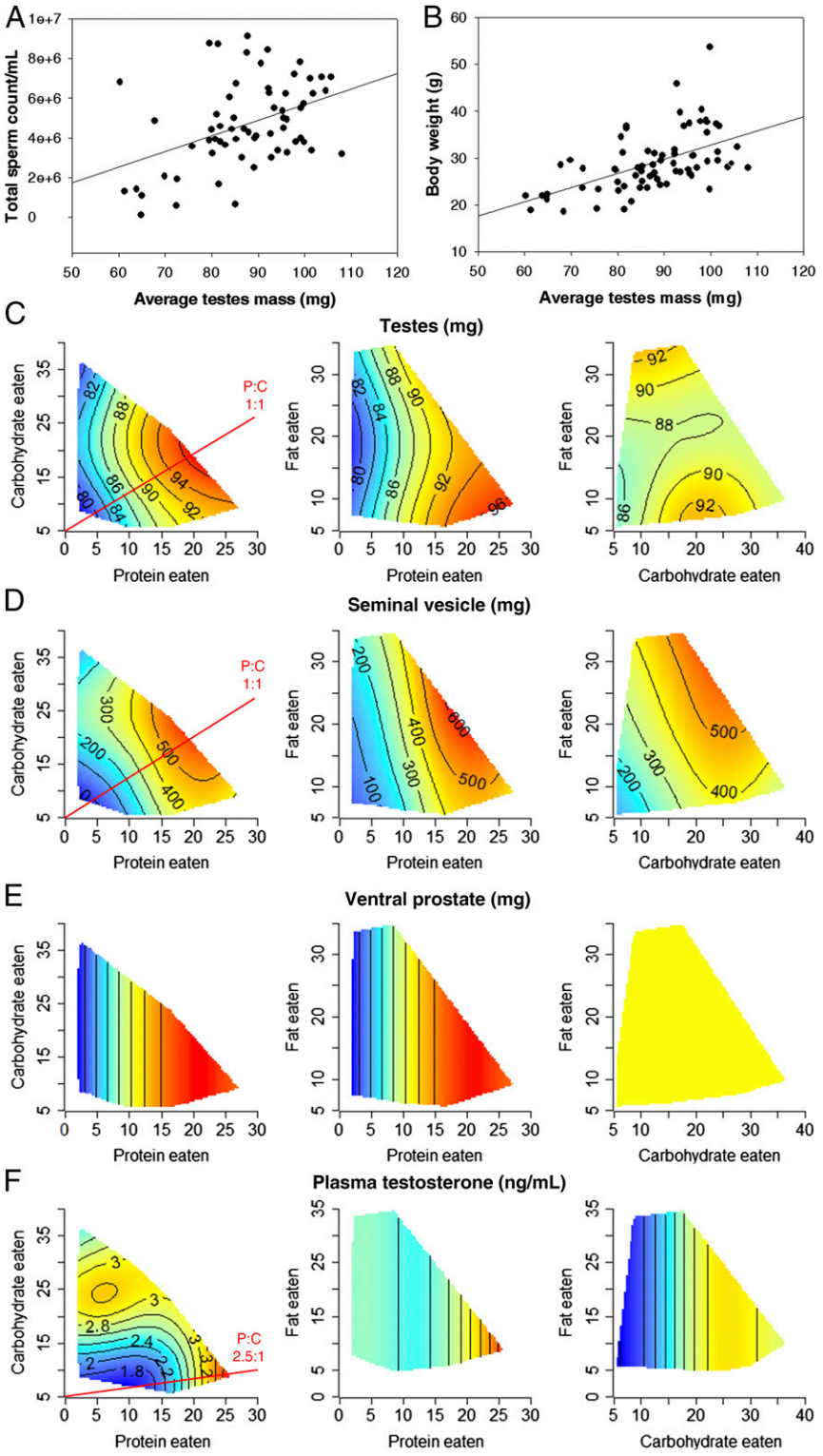

Fig. 3. The effects of macronutrient intake on male reproduction. The relationship between testes mass and (*A*) total sperm count and (*B*) body mass. Response surfaces showing the relationship between macronutrient intake and (*C*) testes mass, as indicators of gametogenesis, and (*D*) seminal vesicle mass and (*E*) ventral prostate mass, as indicators of testosterone production and (*F*) plasma testosterone levels. For response surfaces, the third dimension is sliced at the median. Areas shown in red indicate the greatest value for each response and fall away as the colors shift from red to blue.

reproduction as well as longevity in relation to macronutrients (17). In that study, the sexes differed in their reproductive response to diet composition, with male calling (the single measure of reproductive effort) and lifespan both being greatest on a low P:C diet whereas female lifetime egg production was maximal on a high P:C diet.

It should be noted that values for each of these reproductive variables in mice declined when the P:C ratios were either below or above the optimal value. Although high P:C intakes were linked with better reproductive outcomes, this association was not a linear relationship, and the highest P:C intakes were associated with a decline in reproductive function, as seen previously in invertebrates (5, 17, 41). The results confirm the existence of a macronutrient "target" for reproduction: i.e., that there is an optimum quantity and balance of macronutrients for reproductive function (22, 34). It is notable that most commercial mouse chows have P:C

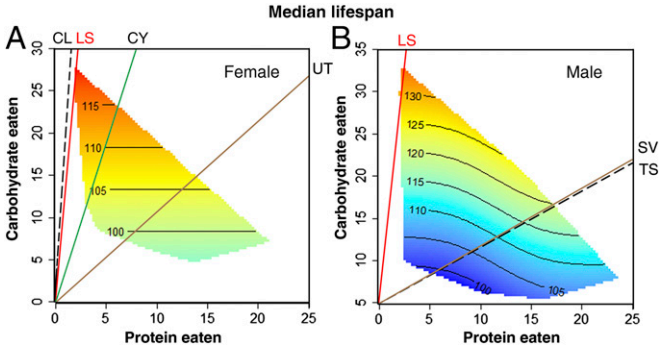

Fig. 4. Median lifespan (LS) and reproductive function are optimized at different macronutrient niches. Response surfaces showing median lifespan of (*A*) females and (*B*) males. The red lines indicate the P:C ratio at which median lifespan is maximized (1:11 and 1:13, respectively). The green solid line indicates the ratio at which the average number of cycling was maximized (CY; P:C 1:8). Black dotted lines represent indicators of gametogenesis, showing either corpora lutea (CL; P:C 1:13) or testes mass (TS; P:C 1:1). Brown lines represent bioassays for steroid-dependent reproductive parameters and show uterus masses (UT; P:C 1:1) for females and seminal vesicle masses (SV; P:C 1:1) for males. For all response surfaces, areas shown in red indicate the greatest value for each response and fall away as the colors shift from red to blue.

ratios around 1:2, which lies between the different values that are optimal for male and female reproductive measures. Experiments have shown that, when given a choice of foods with different concentrations of P and C, mice [outbred Naval Medical Research Institute (NMRI) strain] will choose a P:C ratio in the order of 1:2 (23).

In contrast to the dietary macronutrient compositions that were associated with optimal reproductive function, diets associated with longest lifespan were significantly lower in P:C. We previously reported for this experimental cohort of mice that low P:C intake ratios were associated with longest lifespans (6); here, we show that this pattern was consistent between the sexes, with median lifespan maximized at P:C ratios of 1:11 and 1:13 for males and females, respectively. In *Drosophila melanogaster*, we showed that egg-laying rate was maximized when the P:C ratio was 1:2 and that lifetime egg production was greatest on P:C 1:4 whereas longevity was maximized when the P:C ratio was 1:16 (5). In another species of fly, *Bactrocera tryoni*, lifetime egg production was maximal with a P:C ratio of 1:3 whereas lifespan was maximal at 1:21 (31). These values were derived using parallel GF methodology and are remarkably similar to those we report here in mice. We also previously reported from the same cohort that low P:C intake ratios improved overall late-life cardiometabolic health (6), which may explain why greater CL number was maintained on these diets in older female mice.

In conclusion, our data support the hypothesis that the functional tradeoff between reproduction and longevity arises because there is an optimal balance and intake of macronutrients for reproductive function that differs from that which optimizes lifespan. Compared to the P:C ratio that supports maximal longevity, a higher P:C ratio increases most measures of reproductive function, with this effect differing between males and females. In males, both steroid-dependent responses and indicators of gametogenesis are optimized at a high P:C ratio. Steroid production in females shows a similar pattern; however, gametogenesis seems to be maximized at low P:C ratios, similar to those that maximize lifespan. Our work also supports other studies challenging the primary role of total calories in driving lifespan extension under ad libitum conditions (5, 17, 18, 31, 41, 42) and emphasizes the need for a multidimensional framework to successfully partition the roles of total energy and specific nutrients in reproduction and lifespan (22).

Methods

We have previously published the methods for this study pertaining to diet, lifespan, and late-life cardiometabolic health (6). Here, additional studies were undertaken to evaluate the reproductive tissues of this mouse cohort.

Animals and Diets. Three-week-old C57BL6/J male and female mice were purchased from the Animal Resources Centre and housed in a specific pathogen-free facility at the ANZAC Research Institute. Mice were provided ad libitum access to water and one of 25 experimental diets varying in P, C, F, and E content (Specialty Feeds) (Table S1). To manipulate both nutritional quality and quantity, cellulose was added to the diet, resulting in low, medium, and high energy density diets (8, 13, and 17 kJ·g^{-1}). Food intake was measured weekly for 6 months, followed by monthly measurements and corrections for water content and food spillage. At 15 months of age, 86 male and 97 female mice spanning across all diet groups were anesthetized using a 1:1 ratio of ketamine and xylazine and euthanized, and various tissues were collected for analyses. All experiments were done in accordance with the Sydney Local Health District Animal Welfare Committee (protocol no. 2009/003).

Choice of Measures of Reproductive Function. Two main categories of reproductive parameters were assessed at a single time point in late middle age: gamete and steroid production. In females, gamete production was estimated by follicle and CL counts to measure follicle stock and recent ovulations, respectively, whereas, in the males, we measured epididymal sperm content, which reflects daily sperm production rate in rodents—a standard method from rats used in many species, including humans (43, 44). Steroid production, required for triggering ovulation and preparing uterine microenvironment for embryo implantation and fetal development, was estimated from uterine mass, which is a well-recognized in vivo bioassay for net estrogen exposure (the uterotrophic bioassay) (45). Similarly, in males, to estimate net androgen exposure required to support spermatogenesis and mating, we used seminal vesicle and prostate masses, both long recognized in vivo bioassays for net androgen exposure (the Hershberger bioassay) (46). These endpoints are the basis of traditional whole-animal bioassays for estrogens and androgens and have been revalidated over the last decade by the Organization for Economic Co-operation and Development (OECD) for toxicology research (45, 46).

With the exception of follicle counts, all these reproductive parameters are stable throughout mature murine life so a single measure at 15 months accurately represents lifetime reproductive function. After sexual maturity, ovarian follicle counts decline in a predictable, monoexponential manner, which is fixed for any single mouse strain (47) and in humans (48). On this basis, estimating follicle counts at any one time point (15 months in this case) within a single mouse strain will provide an accurate assessment of the shape of the response surface with respect to dietary treatments and allow extrapolation both backward and forward in time according to the exponential rate function.

Ovary Collection and Follicle Classification and Enumeration. Dissected ovaries were weighed and fixed in 4% (wt/vol) paraformaldehyde at 4 °C overnight and stored in 70% (vol/vol) ethanol before histological processing. Ovaries were then processed through graded alcohols into glycol methacrylate resin (Technovit 7100; Heraeus Kulzer). Ovaries were serially sectioned at 20 μm, stained with periodic acid-Schiff, and counterstained with hematoxylin. Follicle classification and enumeration were based on previously published systems, with modifications (49–51). Three ovaries were analyzed per diet (total of 73 ovaries). To obtain an estimate of growing ovarian follicle populations (small preantral, large preantral, small antral, and large antral) and corpora lutea, total follicle populations were enumerated on three of the largest ovarian cross-sections (≥80-μm intervals) using an Olympus microscope with Stereo Investigator software (MicroBrightField). For analyses, the total number of follicles or corpora lutea per section/ovary (across three sections) was calculated for each animal. For all histological analyses, repetitive counting of follicles was avoided by counting/measuring only follicles containing an oocyte with a visible nucleolus. To avoid bias, all ovaries were analyzed without knowledge of treatment group.

Assessment of Estrous Cycle. Before euthanasia, estrous cycle stage was determined daily for 11 days by light microscope analysis of vaginal epithelial cell smears (28). The stage of the estrous cycle was determined based on the presence or absence of leukocytes and cornified epithelial and nucleated epithelial cells. Proestrus was characterized by the presence of mostly nucleated and some cornified epithelial cells. At the estrus stage, mostly

cornified epithelial cells were present. At metestrus, both cornified epithelial cells and leukocytes were present. At diestrus, primarily leukocytes were visible.

Homogenization-Resistant Sperm-Head Count. Excised epididymis were stored frozen at −80 °C. Thawed cauda epididymis was excised from the corpus, weighed, and homogenized in 1 mL of PBS (52, 53). Homogenate was diluted as needed to count homogenization-resistant sperm head (stage 14–16 spermatids) in a Neubauer hemocytometer.

Statistical Modeling and Analysis. Median lifespan was analyzed using Kaplan–Meier survival analysis in Sigma Plot. Macronutrient, energy, and food intake were analyzed based on average intake per mouse per cage per day. All other responses are modeled for individual animals. Data are presented using the GF approach and analyzed using generalized additive modeling (GAM). For data using counts (follicle number, CL number, and number of estrous cycles), GAMs were fitted with the negative binomial distribution. The 2D response surfaces plotted at the median of the third axis are visualized using thin-plate splines in R (v 3.0.2) (6).

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