Drosophila Feeding Behavior and Demographic Mechanisms of Lifespan Extension

Thesis by

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Abstract

Why do we age? Most organisms undergo senescence, a process involving progressive functional decline culminating in death, yet this widespread phenomenon remains largely mysterious. A number of genetic and environmental factors affect longevity, the best conserved and most widely studied of which is dietary restriction (DR), a reduction of nutrient intake short of malnutrition. Since nutrient ingestion determines lifespan, any factor affecting longevity—particular food components, genetic pathways or drugs—may do so indirectly, by altering feeding behavior. This is particularly true in *Drosophila*, which is normally kept in conditions where food is present in excess. Moreover, since DR is applied by aging flies on two different food concentrations—diluted media are associated with an extended lifespan—animals may alter their intake in response to the change in nutrient content. Since the medium is also the only water source, this compensatory feeding would result in changes in hydration, introducing a second experimental variable. Despite these issues, *Drosophila* feeding behavior has classically been ignored or superficially characterized in the context of aging research, partly due to the absence of appropriate methodology. We developed two complementary assays allowing long-term measurement of food intake. Using these techniques, we present the first characterization of real-time *Drosophila* feeding behavior. Our results reveal gender-specific feeding trends and show that mating stimulates female appetite via the seminal Sex Peptide. Additionally, we show that ingestion is dramatically affected by food dilution or dietary additives. Animals fed concentrated media restrict their intake

and are chronically thirsty. We have found that lifespan extension by classical DR paradigms is abolished in the presence of ad libitum water, challenging the long-held assumption that DR affects longevity by altering nutrient intake. We characterize a new regime that robustly prolongs lifespan irrespective of water availability, and thus likely represents a more relevant model for mammalian DR. In contrast to previous claims, demographic analysis using this paradigm indicates that DR acts not by reducing the immediate risk of death, but by slowing the accumulation of age-related damage. Our findings directly challenge current views on the mechanistic basis of DR and have broad implications for the study of aging and nutrition in model organisms.

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Thesis Overview

The work in this thesis covers our development of two assays of *Drosophila* feeding behavior and the application thereof to several questions in aging and behavior research.

The Introduction reviews the history of aging research in model organisms, with particular focus on dietary restriction (DR), the *Drosophila* system, and its particular experimental issues.

Chapter 1 includes a paper describing the first feeding assay—radioactive isotope labeling – and its application to address the issue of compensatory feeding. Our results show that flies compensate for medium dilution by adapting their intake.

Chapter 2 includes a paper where, using radioactive isotope labeling, we illustrate gender differences in feeding behavior and show that mating stimulates female feeding via the action of the Sex Peptide.

Chapter 3 includes a paper describing the second feeding assay—the CAFE—with which we characterize real-time feeding behavior and demonstrate the effect of dietary drugs on ingestion.

Chapter 4 includes a paper where we show that providing ad libitum water can abolish lifespan extension by classical DR paradigms. This finding casts serious doubt on the relevance of most of the work on *Drosophila* DR. We also characterize a new DR regime that affects lifespan irrespective of hydration and propose it as a better model of mammalian DR.

In Chapter 5, we present unpublished data reexamining the effect of DR on mortality rate. We show that previous claims are also attributable to hydration, and thus likely irrelevant to understanding mammalian mortality. In conditions where water availability is not a factor, DR affects longevity by slowing the accumulation of age-related damage, in direct contradiction to current belief.

Chapters 6 and 7 include papers describing findings made in the process of our experiments, but that are tangential to the central focus of our research. Chapter 6 includes a paper where two novel peptides, including Sex Peptide, are shown to activate the G protein-coupled receptor Methuselah in a cell culture system. Chapter 7 includes a paper where we describe a technique for genotyping single flies that bypasses some of the limitations of current methodology.

The conclusion summarizes the significance of our findings for the study of aging and behavior in flies and discusses future directions for these fields.

In final remarks, I describe some of the lessons I learned in graduate school and how they affect my perspective on scientific research.

The appendix presents data on the effect of reactive oxygen species (ROS) on female sexual behavior. We show that several interventions altering ROS levels control egglaying and sexual receptivity in virgins. Specifically, our data indicate that ROS may act in neurons to modulate behavior.

Introduction

1. Aging

Aging is defined as an incremental functional and reproductive decline associated with increased mortality (1). A slew of evolutionary theories of aging have been formulated, most of which have in common the idea that senescence is an evolutionary "accident," the result of a lack of natural selection in favor of late survival (2). Most animals in the wild die of environmental causes at a relatively early age (3), and thus never experience senescence, making old age evolutionarily irrelevant. As a result, mutations favoring late survival are not selected for, whereas those causing functional decline after the natural life expectancy are not selected against, and may even be selected for if they also confer an early benefit – a phenomenon known as antagonistic pleiotropy (4).

Age-dependent decay is not an inevitable characteristic of life. For example, aging appears to be tissue specific. Unlike somatic cells, the germline suffers no irreversible age-dependent decline and is essentially immortal (5). Old organisms beget young ones, and we are the product of a long line of cell divisions dating back to our early evolutionary ancestors. Moreover, aging seems to occur only in species with an identifiable germline, whereas those reproducing, for example, by fission, show no increase in mortality with age (6–8). Based on these observations, the disposable soma theory proposes that organisms must distribute their limited metabolic resources between somatic maintenance and reproduction, and tend to favor the latter, while somatic investment is sufficient to reach maturity and reproduce effectively, but is neglected thereafter, causing the decline we know as aging (1). One tantalizing

prediction of this theory is that we have the physiological ability to slow senescence, possibly at the expense of fertility. In support of this notion, germline ablation extends the lifespan of both worms and flies (9–11). Moreover, the somatic cells of long-lived, mutant *C. elegans* show a germline-like pattern of gene expression, and this characteristic is necessary for their longevity (5).

2. Model Systems of Aging

Biology has long relied on model organisms to dissect and manipulate lifespan. Early in the 20th century, *Drosophila* longevity was found to be sensitive to a number of environmental factors, including temperature, food composition, population density and mutagens (12). At around the same time, food restriction was shown to extend lifespan in rodents and fish (13). In the decades that followed, this effect, classically referred to as caloric restriction (CR), was also found to delay the onset of age-related pathology (13). We now know that CR can prolong survival in several rodent species, protozoa, rotifera, yeast, worms, flies, spiders, fish, dogs and probably non-human primates (14).

Model organisms have also been instrumental in identifying genetic pathways controlling lifespan. A large number of single-gene mutations conferring lifespan extension have been identified in yeast, worms, flies and mice. Particularly well-conserved players include the insulin-like and target of rapamycin (TOR) pathways and the *silent information regulator 2* (*Sir2*) gene (15). Interestingly, these genes have also been found to play a role in CR (15).

3. Food restriction and *Drosophila* lifespan

Among the factors found to influence *Drosophila* longevity, food restriction—which in flies is known as dietary restriction (DR)—has received particular attention. The large evolutionary distance from yeast to humans and the long lifespan of rodents make flies and worms particularly attractive models to study aging. Like *C. elegans*, flies are small and easy to maintain in large numbers, but unlike nematodes, *Drosophila* has a full nervous system and gene expression can be easily manipulated with tissue specificity.

DR was first shown to prolong fly survival in 1996 (16). The original paradigm, used to this day by most researchers in the field, consists of aging flies on two different food concentrations. This simple food dilution protocol raises a number of experimental issues (17). First, flies may compensate for dilution by regulating their intake – compensatory feeding (CF). Second, if CF does occur, flies on different foods will ingest different amounts of water, since the medium is the only source of both food and water. Third, richer media may stimulate bacterial and/or fungal growth. Thus, any effect of food dilution may be due to (i) differential nutrient intake, if there indeed is one, (ii) changes in hydration or (iii) infection.

3.1 Measuring feeding in Drosophila

At the time we initiated our work, in 2004, fly DR experiments typically did not include quantification of food intake, and CF was simply assumed not to occur.

Yet, CF had been shown in several insect species (18, 19) and some reports suggested its occurrence in *Drosophila*, although the findings were based on atypical dietary conditions (20) or indirect assays (21).

Measuring ingestion in fruit flies is not trivial, due to their small size. A number of studies have relied on indirect methods. Egg-laying rate (22), fecal pellet number (21) and the fraction of individuals feeding at a given time (23, 24) have all been suggested as surrogates for ingestion measurements. While these parameters may sometimes correlate with intake (25), it is obvious that trying to infer food consumption from such assays is far from ideal, for three reasons. First, they are much less sensitive to feeding changes than direct measurements. Second, a change in feeding rate does not necessarily result in a change in fecundity, fecal pellet number or time spent feeding. And third, surrogate assays can be misleading when studying the effect of mutations, drugs or environmental conditions, since these factors may directly affect the surrogate parameter, irrespective of feeding rate.

Up to 2004, two direct assays of intake had been described in fruit flies. The most popular method involves labeling the food with visible dyes (20). These compounds are not absorbed and accumulate in the gut for the first 40–50 minutes of exposure, after which egestion commences and dye levels no longer accurately reflect ingestion (26). Moreover, because satiated flies will normally not eat in a short time window, dye assays are commonly preceded by a starvation period. Finally, animals are startled when transferred onto a fresh medium and may not stabilize until after the

assay window. For these reasons, dye labeling is only accurate for short-term ingestion and does not reflect steady-state feeding.

On the other hand, radioactive food labeling, which had remained essentially unused, allows cumulative recordings up to several days (27), but has caveats of its own. In addition to the hazardous nature of the reagents, isotope levels reflect not only ingestion, but also absorption, metabolization and excretion of the label (28).

In conclusion, there was a clear need for improvement in the methodology used to assay *Drosophila* feeding, a crucial parameter for the study of aging, metabolism, growth and behavior. This challenge constitutes the starting point for our work.

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Chapter 1

Compensatory Ingestion upon Dietary Restriction in *Drosophila melanogaster*

This chapter consists of a paper coauthored by Pankaj Kapahi and Seymour Benzer, which appeared in *Nature Methods*. This publication describes our optimization of a quantitative feeding assay using isotope radiolabeled-food medium. The work was our first effort to address the need for monitoring feeding behavior of animals exposed to different media, such as in the DR paradigm. We show that, in contrast to long-held assumptions and previous claims, flies compensate for food dilution, a fact that must be taken into account in *Drosophila* aging research.

Compensatory ingestion upon dietary restriction in *Drosophila melanogaster*

Gil B Carvalho, Pankaj Kapahi & Seymour Benzer

Dietary restriction extends the lifespan of numerous, evolutionarily diverse species[1]. In *D. melanogaster*, a prominent model for research on the interaction between nutrition and longevity, dietary restriction is typically based on medium dilution, with possible compensatory ingestion commonly being neglected. Possible problems with this approach are revealed by using a method for direct monitoring of *D. melanogaster* feeding behavior. This demonstrates that dietary restriction elicits robust compensatory changes in food consumption. As a result, the effect of medium dilution is overestimated and, in certain cases, even fully compensated for. Our results strongly indicate that feeding behavior and nutritional composition act concertedly to determine fly lifespan. Feeding behavior thus emerges as a central element in *D. melanogaster* aging.

Defined as a reduction in nutrient intake without malnutrition, dietary restriction prolongs the life of species as diverse as nematodes, insects and mammals[1,2], with preliminary results indicating that this effect may be conserved in primates as well[3,4]. In rodents (where it is commonly known as caloric restriction), dietary restriction also prolongs vitality and delays the onset of age-associated diseases such as cancer and cardiovascular pathology[5,6]. Animals subjected to chronic dietary restriction exhibit multiple physiological changes, including reduced glucose, insulin and insulin-like growth factor 1 (IGF-1) blood levels, increased insulin sensitivity and overall dampened inflammatory response[6]. In addition, studies in human subjects suggest that dietary restriction may positively impact critical health factors such as blood pressure and glucose and cholesterol blood levels[7–9]. Despite the obvious biomedical relevance of research on dietary restriction, seven decades of work have conveyed little mechanistic insight. In particular, and as a result of the wide variety of methods used for dietary restriction application in different model organisms, it remains unclear whether the evolutionarily conserved beneficial effect is exerted through a common physiological mechanism.

In both nematodes and rodents, dietary restriction heavily relies on patterns of feeding behavior. In *Caenorhabditis elegans*, where pharyngeal pumping rate serves as an indirect measure of food intake[10,11], the most common method of dietary manipulation takes advantage of animals defective in pharyngeal constriction — the *eat* mutants[12]. The food source, the bacterium *Escherichia coli*, is provided in abundance, but ingestion is limited by the neuromuscular defect of the mutants. In experiments with rodents, the 'restricted' group is fed a fraction (typically ~65%) of the food consumed by the *ad libitum* group[2]. Therefore, in both of these model systems dietary restriction relies on a bona fide reduction of nutrient intake. In contrast, dietary restriction in *D. melanogaster* typically involves simple dilution of the food medium[13,14]. This procedure, as a rule, is not accompanied by direct quantitation of intake, neglecting potential changes in ingestion leading to partial or total nutritional compensation. Compensatory feeding in response to changes in food composition has been described in several insect species[15,16]. In *D. melanogaster*, however, partly owing to differences in methodology, no consensus has been reached regarding this issue, and

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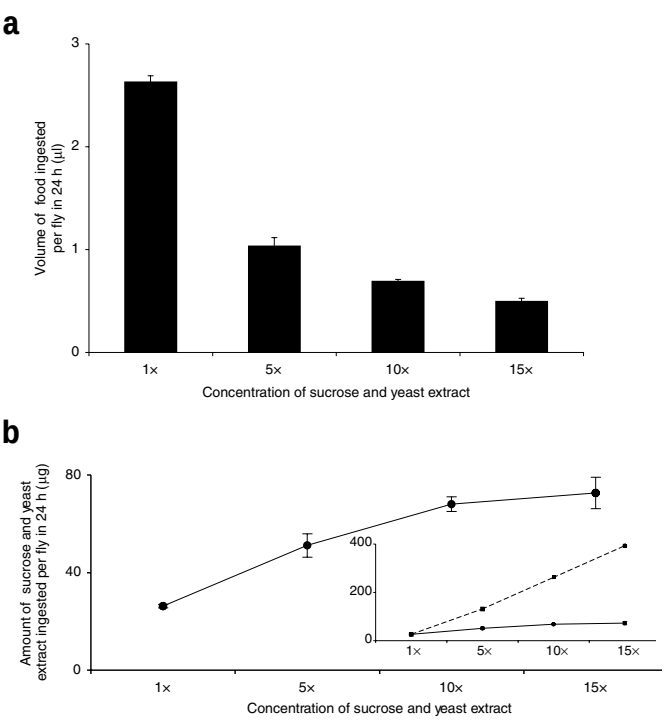

Figure 1 | Regulation of feeding behavior in response to dietary modulation. (**a**) Volume of food ingested per fly over 24 h on four different medium concentrations at 25 °C (mean ± s.d. of four replicate samples of 15 females each). Unpaired, two-tailed *t* tests: 1× versus 5×, $P = 0.0001$; 5× versus 10×, $P = 0.0005$; 10× versus 15×, $P = 0.0003$ (**b**) Net sucrose and yeast extract intake on the four nutritional conditions, in micrograms ingested per fly per 24 h (mean ± s.d.). Inset, actual nutrient intake (solid line) markedly differs from expected intake based on medium concentration only (dashed line).

the general assumption underlying dietary restriction studies is that compensation is negligible or does not occur. Previous work suggests that fruit flies can sense sucrose concentration and accordingly regulate intake[17,18], but the conditions used in these studies differ markedly from the customary laboratory media used for raising and aging flies. Indirect measures, such as fecal pellet density, also indicate that nutrient dilution can produce compensatory feeding[19]. In contrast, a recent report asserts that dietary manipulation elicits essentially no compensatory ingestion, based on the fraction of animals with their proboscis contacting the food at a given time, but without any measurement of actual intake[20].

D. melanogaster is a particularly valuable model for the study of the interaction between nutrition and mortality, having yielded some of the most important recent advances in our understanding of the effects of dietary manipulation. It is essential that the methodology of dietary restriction application be consistently established if the mechanisms of lifespan extension by nutrient modulation are to be elucidated in this model system. By using a method to directly monitor *D. melanogaster* feeding behavior, we demonstrate that dietary restriction elicits dramatic changes in the volume of food ingestion that can compensate for differences in medium concentration, making the latter a misleading value when considered in isolation. In addition, our findings indicate that the lifespan of *D. melanogaster* is not exclusively determined by food source composition, but rather it is the product of the interaction between nutrient availability and active feeding behavior.

Dietary restriction elicits dramatic compensatory feeding behavior

Isotope labeling of the food medium allows for sensitive and specific quantitation of intake. We determined adult feeding rate in four dietary regimes over 24 h by incorporating a [α-^{32}P]dCTP tracer in the fly food. Signal incorporation was near-linear up to 72 h (data not shown). The four media were based on a binder of 8% cornmeal, 0.5% bacto agar and 1% propionic acid, with added sucrose and yeast extract at defined concentrations. We defined 1× as 1% sucrose + 1% yeast extract (see Supplementary Methods online). Nutrient dilution had a striking impact on volume of food intake (Fig. 1). Flies maintained on 5×, 10× and 15× regimes ingested, respec-

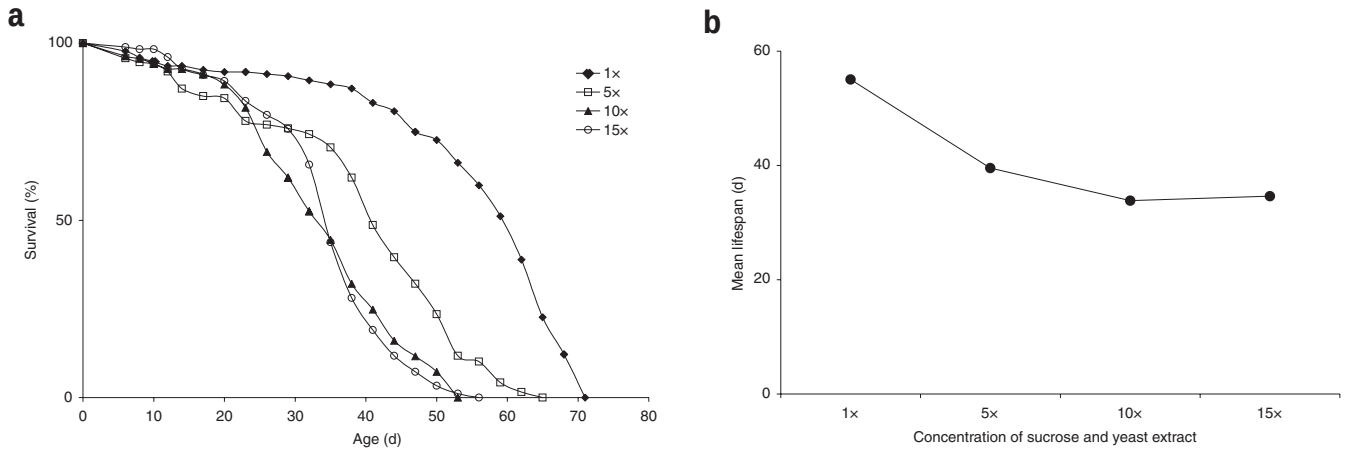

Figure 2 | Feeding behavior influences *D. melanogaster* lifespan. (**a**) Survival for virgin females at 25 °C on four different nutritional concentrations. Longevity correlates with actual food intake. (**b**) Mean lifespan as a function of medium concentration. Survival on 5× is 28% shorter than on 1× (logrank test, $P < 0.0001$, $\chi^2 = 134.8$), and 17% longer than on 10× (logrank test, $P < 0.0001$, $\chi^2 = 30.72$), whereas lifespan on 10× and 15× does not differ significantly (logrank test, $P = 0.7993$, $\chi^2 = 0.06466$). 1×, $n = 172$, mean = 55 d; 5×, $n = 187$, mean = 40 d; 10×, $n = 137$, mean = 34 d; 15×, $n = 178$, mean = 35 d.

tively, 2.6, 3.8 and 5.4 times less volume than animals on 1×. We obtained identical results with three alternative tracers: [^{14}C]leucine, [^{14}C]sucrose and [α-^{32}P]dATP (data not shown). Both the absolute values and the ratios between differently-fed groups were remarkably reproducible, both within (Fig. 1a) and across experiments, indicating that appetite is surprisingly constant under each set of dietary conditions and tightly regulated in response to food changes. Notably, our measurements of isotope incorporation reflect nutrient assimilation rather than simple ingestion and may thus be the most pertinent value to studies of metabolism and physiology.

We determined the amount of sucrose plus yeast extract ingested over 24 h (Fig. 1b). The result markedly contrasts with expected values based on nutrient concentration alone (Fig. 1b, inset). For instance, enriching the medium from 1× to 5× resulted in less than a twofold increase in nutrient uptake, and flies on 10× consumed only 33% more nutrients than animals on 5×. Most strikingly, raising food concentration from 10× to 15× did not alter actual nutrient intake. It is also worth noting that, between 5× and 15×, regimes similar to the ones commonly referred to, respectively, as "dietary restriction" and "control"[20], and generally assumed to represent a 200% enrichment, the observed actual difference in nutrient intake was only 40%. These results demonstrate the existence of a behavioral mechanism allowing *D. melanogaster* to actively compensate for differences in food source composition, and call for a reassessment of the protocols used for dietary manipulation in this species.

Feeding behavior influences lifespan

We hypothesized that feeding behavior is a central determinant of longevity. We therefore expected the lifespan of flies aged on the different regimes to parallel nutrient ingestion rate, rather than the composition of the medium alone. In fact, survival on 10× and 15× food did not differ significantly ($P = 0.8$; Fig. 2). This is in full agreement with our measurements of actual nutrient intake (Fig. 1b) and clearly contradicts the expectation based on medium dilution (Fig. 1b, inset). Moreover, as illustrated by the symmetry of the two curves in Figures 1b and 2b, mean lifespan correlated tightly with nutrient intake, but not with food concentration (Fig. 1b, inset). Our findings are consistent with the hypothesis that actual nutrient intake is a central determinant of lifespan in flies subjected to dietary manipulation, whereas medium dilution, considered in isolation, is not a reliable parameter.

Conclusion

Our findings draw attention to the importance of monitoring a behavioral element in *D. melanogaster* longevity studies, particularly those involving dietary manipulation. Much like lifespan, any biological process depending heavily on nutrition is likely to be the result of a fine balance between two elements, one passive—food composition—and one active—feeding behavior. Other fields in which nutrition is an essential factor (for example, growth, reproduction and obesity) should therefore equally benefit from careful characterization of the role of fly appetite. Although feeding rates are likely to vary under different laboratory conditions, the magnitude and reproducibility of the effect described here strongly suggests a conserved phenomenon. It will be of particular interest to determine the conditions under which appetite compensation is partial or complete. Further work will also be required to determine the role of individual food components in appetite regulation.

Adaptation of feeding behavior to nutrient source composition has an important ecological role in the wild. In the presence of plentiful and highly nutritious food, it is of evident advantage to limit intake. Conversely, when nutrient sources are poor or scarce, flies will benefit from ingesting larger meals. Elucidation of the physiological and molecular bases of appetite modulation in *D. melanogaster* may bear relevance to understanding such pathologies as obesity and feeding disorders.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Supplementary Methods

Feeding assay. Virgin females (2–3 day-old) were conditioned for 4 days to the specific nutritional regime to be tested (15 animals/vial). Flies were then transferred to the same medium containing 1.4 mCi/L dCTP [α–^{32}P] (MP Biomedicals, Irvine, CA) and allowed to feed for 24 h, then transferred to empty vials to groom for 30 min (to ensure removal of any cuticular radioactive deposits), anesthetized by cold and assayed in 10 mL scintillation fluid (Research Products International, Mount Prospect, IL), using a Beckman LS 5000 TA Liquid Scintillation System. Each experiment included two standards with dCTP [α–^{32}P] diluted in water (1:10,000 volume:volume), which were used to perform the conversion from scintillation counts to food volume. Signal incorporation over both the 24 h test period and an extended 72 h period was near linear.

Food media. Flies were raised to adulthood on Lewis medium (*Drosophila Information Service* 34, 117). The media employed for dietary manipulation consisted of varying concentrations of sucrose and autolyzed yeast extract (Bacto Yeast Extract, B.D. Diagnostic Systems, Franklin Lakes, NJ) diluted in a binder of 8% cornmeal, 0.5% bacto-agar and 1% propionic acid. 1× food was defined as 1% sucrose + 1% yeast extract.

Lifespans. Virgin females were collected under brief carbon dioxide anesthesia. Lifespans were conducted with ~40 animals per vial at 25^{o}C on a 12 h/12 h light/dark cycle. Fresh food was provided and deaths were scored every 2 or 3 days. Data were analyzed using Graphpad prism software.

Chapter 2

Allocrine Modulation of Feeding Behavior by the Sex Peptide of *Drosophila*

This chapter consists of a paper coauthored by Pankaj Kapahi, David J. Anderson and Seymour Benzer, which appeared in *Current Biology*. Using radiolabeling, we show that mating stimulates female feeding rate and that this phenomenon is mediated by the Sex Peptide, thus adding a novel behavioral component to the *Drosophila* postmating response.

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Report

Allocrine Modulation of Feeding Behavior by the Sex Peptide of *Drosophila*

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Summary

Mating elicits a dramatic reprogramming of female behavior in numerous insect species. In *Drosophila*, this postmating response (PMR) comprises increased egg-laying rate and reduced sexual receptivity and is controlled by the products of the male accessory glands, a family of ~80 small peptides transferred in the male seminal fluid [1–9]. Here, we show that copulation strongly stimulates female food intake. Remarkably, this change is abolished if the males lack a single, small seminal protein, the Sex Peptide (SP). Ectopic expression of SP in virgin females mimics the effect of mating on feeding behavior, demonstrating that SP is the main agent controlling this behavioral paradigm. Our observations identify enhanced feeding behavior as a novel component of the *Drosophila* PMR and suggest that SP represents a molecular link between energy acquisition and reproductive investment.

Results and Discussion

Nutrient availability plays a critical role in reproductive success [10–12]. Accordingly, changes in patterns of feeding behavior correlate with reproductive status in a wide range of organisms [13–15]. However, the mechanisms regulating this vital process are not well understood. To investigate this issue, we recorded adult food intake by allowing flies to feed on medium colored with a nonabsorbable, nonmetabolizable dye [16]. Visual inspection revealed a striking effect of mating status on female abdominal food accumulation. Mated females ingested substantially larger meals than age-matched virgins (Figure 1A). This disparity was both accentuated and accelerated if a 12 hr starvation period preceded the feeding trial. Spectrophotometric quantitation showed that, in these conditions, mated females consumed ~2.3 times as much food as virgins (Figure 1B). Other dyes of different colors and chemical compositions gave similar results (data not shown).

Drosophila feeding behavior can be monitored by radioactive labeling of the medium [17, 18]. An essential advantage of this method lies in its enhanced specificity and sensitivity, which make it possible to record steady-state food consumption in nonstarved flies. In addition, food intake can be measured over longer periods, avoiding short-term fluctuations and circadian variation. We recorded adult food ingestion over a 24 hr period by using food labeled with [α-^{32}P]dCTP. Averaged across multiple, independent trials, ad-libitum-fed, mated females showed a 56% elevation in radioactive tracer level when compared to virgins (Figure 1C). This result was reproducible with different isotopes ([α-^{32}P]dATP, [^{14}C]sucrose) and was conserved across several wild-type strains, including Canton-S, Oregon R, and Dahomey (data not shown). This observation cannot be explained simply by an enhanced total food capacity of mated animals, because isotope incorporation in both physiological states continued to increase up to at least 72 hr on labeled food (Figure S1 in the Supplemental Data available online). The 24 hr measurements shown in Figure 1C are therefore far from reaching saturation. Furthermore, the higher tracer levels in mated females are not a consequence of defective or delayed nutrient metabolization and/or excretion, given that the isotope level declined significantly faster in mated females than in virgins after the 24 hr pulse of labeled medium (Figure S2). This disparity in the rate of isotope elimination may reflect incorporation into developing oocytes in mated females. Together with the results obtained with dye-colored food (Figures 1A and 1B), these findings strongly suggest that our measurements reflect bona fide differences in volume of food ingestion between the virgin and mated states. In contrast to the situation in females, male feeding was not affected by mating status (Figure 1D). These results identify enhanced feeding behavior as a novel component of the *Drosophila* PMR.

Both previously described elements of the behavioral PMR—egg laying and rejection of secondary copulation—are regulated by the products of the male accessory gland [5]. We therefore asked whether the accessory-gland proteins (Acps) are also responsible for the feeding-behavior changes in mated females. Genetic ablation of the accessory-gland main cells can be achieved through expression of a modified form of diphtheria toxin subunit A (DTA) under the control of the main cell-specific promoter Acp95EF [5]. These DTA-expressing males produce only vestigial amounts of Acps (~1% of wild-type) and induce no egg-laying and only a slight, transient reduction of female receptivity [5]. Females mated to DTA males displayed no elevation of food intake, whereas isogenic control males lacking the DTA construct induced a normal response (Figure 2A), indicating that the physiological stimulation of feeding behavior requires the Acps.

One Acp in particular, the Sex Peptide (SP), is both necessary and sufficient to induce the PMR in virgins [6–9]. We therefore asked whether SP is the particular Acp responsible for stimulating female food intake. SP^0 males, which specifically lack SP as a result of a targeted chromosomal deletion, but normally express and transfer all remaining Acps and sperm [8], failed to

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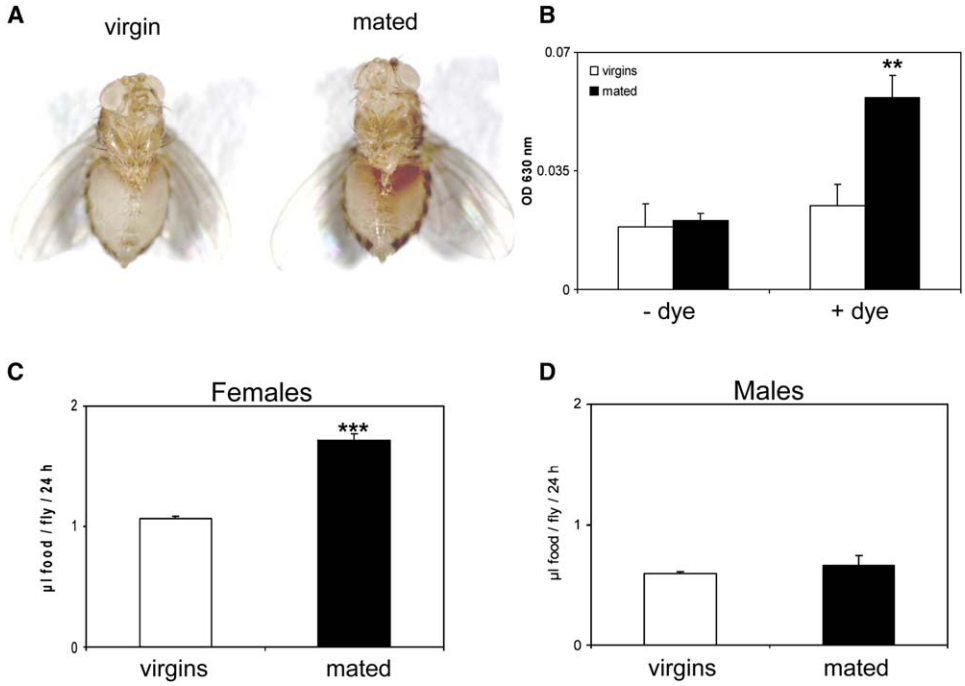

Figure 1. Mating Stimulates Female Food Intake

(A) Virgin and mated females after feeding on red-dyed medium for 2 hr.

(B) Feeding rate of virgin (− dye, n = 42; + dye, n = 39) and mated (− dye, n = 40; + dye, n = 36) females allowed to feed on medium with or without red dye for 30 min following 12 hr of wet (water-only) starvation. Shown is the average per fly ± standard deviation (SD) of three replicates. Y axis represents values of optical density (OD) of abdomen homogenate (assayed at 630 nm).

(C and D) Induction of feeding rate upon mating is female specific. Ingestion volume of [α-32P]dCTP-labeled food over a 24 hr period by ad-libitum-fed virgin and mated females (C) and males (D). Results are expressed as volume of food intake (in µl) over 24 hr averaged per fly ± SD of four replicates of 15 animals per condition. ** indicates $p < 0.01$; *** indicates $p < 0.0001$, two-tailed t test.

significantly induce feeding in females (Figure 2B). Both DTA and SP^0 males showed courtship and mating rates similar to those of the respective controls and successfully fertilized all females they were kept with, as assayed by scoring viable progeny of females kept in individual vials (data not shown). These results demonstrate that the main-cell Acps, and SP in particular, are required for stimulation of postcopulatory food ingestion in females.

We next tested directly the action of SP in regulating female feeding behavior. Ectopic expression of SP in the adult fat body of virgin females by means of a yolk protein 1 enhancer (*yp1*) has been shown to be sufficient to induce the two classical components of the PMR [7]. Females bearing the *yp1*-SP fusion construct exhibited a constitutively increased feeding rate that was not further elevated by mating (Figure 3A), suggesting that SP can, by itself, elicit a mated-like appetite in virgins. We tested this hypothesis further by expressing SP under the control of an upstream-activating system (UAS) promoter. Previous work has identified several independent galactose 4 (GAL4) insertion lines that, when used to drive SP, can elicit the PMR in virgin females [19]. Indeed, expression of SP under the control of either the 9Y- or C370-GAL4 driver lines [19] markedly stimulated virgin feeding rate (Figures 3B and 3C). Importantly, in neither case did copulation further increase food ingestion. Three additional GAL4 drivers gave identical results (data not shown). Although the central nervous system is the only area in common among the expression patterns of the five driver lines ([19], G.B.C. and S.B., unpublished data), the fact that SP is expressed as a secreted, diffusible molecule precludes a definite conclusion concerning its site of action. These findings demonstrate that SP modulates postcopulatory feeding in females, whereas sperm and the act of copulation per se do not play substantial roles.

In numerous animal species, including humans, enhancing nutrient acquisition is a common strategy accompanying reproductive effort, and its pivotal role in ensuring reproductive success is well established [10–12]. *Drosophila* has found an elegant and effective way to couple reproductive investment to increased acquisition of energy resources—a single, small peptide transferred in the male ejaculate. Peptides play a central role in appetite control, both in insects and in higher organisms [20–24]. Remarkably, in this case, the molecule is produced by and regulates the feeding behavior of two separate individuals. Sexual allocrine mechanisms have also been described in vertebrates. For example, prostaglandins secreted in human semen can modulate female immune response [25], a role that has also been attributed to the SP of *Drosophila* [26]. How does SP orchestrate such a dramatic behavioral and physiological reprogramming? In the case of appetite modulation, a possible mechanism is suggested by the fact that SP binds to the subesophageal ganglion [27], a neuronal center previously implicated in taste recognition and feeding [28, 29]. Alternatively, SP may regulate food intake indirectly. Ex vivo, SP acts on the corpus allatum

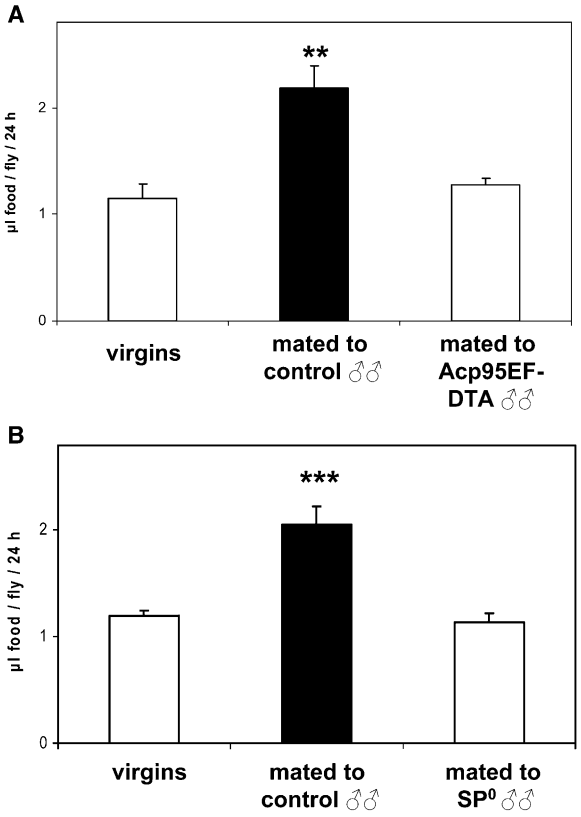

Figure 2. SP Is Necessary for Postcopulatory Induction of Female Food Intake

(A) Genetic ablation of male accessory-gland main cells abolishes stimulation of feeding behavior. Experimental males carry a construct in which the Acp95EF main-cell promoter is fused to the modified coding sequence of diphtheria toxin subunit A (DTA) [5]. Control males have identical genetic background but do not bear the DTA construct (one-way ANOVA, p = 0.0021).

(B) Males that lack SP (SP^0) but produce and transfer normal amounts of remaining Acps and sperm fail to stimulate female appetite. Experimental males carry the null mutant allele SP^0 (introduced by homologous recombination) over the Δ^{130} deletion, which uncovers the *SP* locus. Control males have identical genetic background but carry a wild-type copy of *SP* over Δ^{130} [8] (one-way ANOVA, p = 0.0001). All values are expressed as volume of food intake (in µl) over 24 hr averaged per fly ± SD of three replicates of 15 animals per condition.

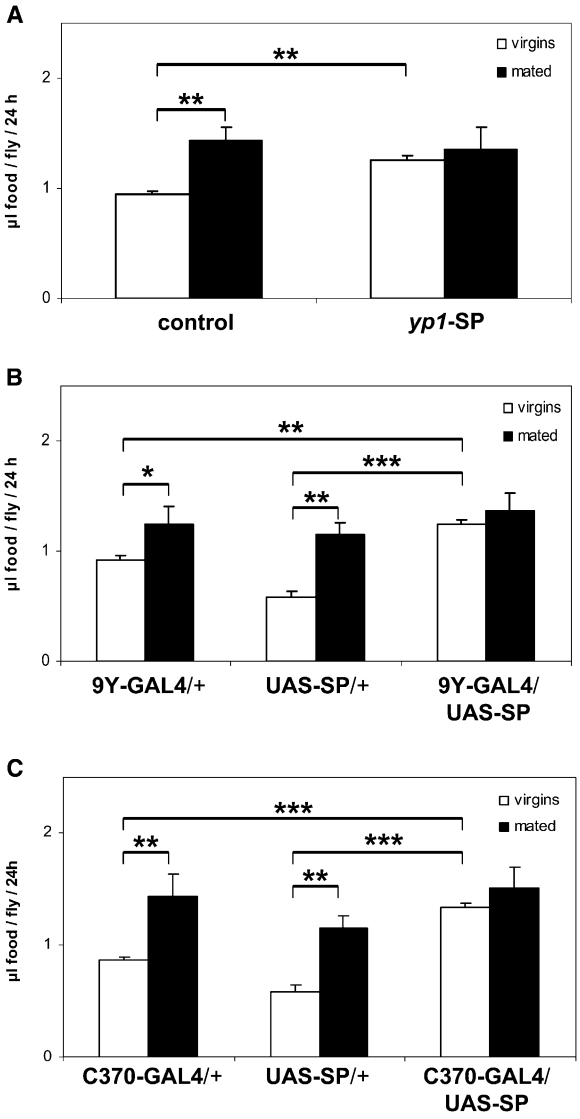

Figure 3. Ectopic Expression of SP in Virgin Females Mimics the Effect of Copulation

(A) Constitutive fat-body expression of SP in virgins by means of the yolk protein 1 (*yp1*) enhancer stimulates feeding to mated-like levels. Isogenic control strain is *cinnabar; rosy* (*cn; ry*).

(B and C) Effect on feeding behavior of expression of a UAS-SP construct driven by the 9Y- (B) or C370- (C) GAL4 driver lines. All values are expressed as volume of food intake (in µl) over 24 hr averaged per fly ± SD of three replicates of 15 animals per condition. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.0001$, two-tailed t test.

to stimulate the secretion of Juvenile Hormone (JH) [30], which plays a crucial role in sexual maturation and oogenesis in *Drosophila* females [31–33]. Induction of oogenesis and vitellogenesis by JH may in turn induce female food intake. In this regard, it will be interesting to investigate whether appetite modulation requires intact reproductive activity.

Our findings raise another intriguing question. Mating drastically reduces the lifespan of *Drosophila* females [34], a phenomenon that has been attributed to the action of the Acps [35], and to SP in particular [36]. Given the link between increased food consumption and shortened lifespan in many organisms, it is conceivable that the reduced longevity of mated females may somehow relate to their accrued nutrient ingestion. Further study on the biology of Acps should help elucidate this intriguing aspect of animal reproduction.

Experimental Procedures

Drosophila *Strains*

Unless otherwise stated, flies were in the *w1118* background. Acp95EF-DTA (mc/DTA-D) was kindly provided by M. Wolfner. SP^0 and control stocks were kindly provided by E. Kubli. UAS-SP was kindly provided by T. Aigaki. *yp*1-SP (Yp1-hsp70-SPgene) and control stocks were obtained from the Bloomington Stock Center.

Culturing Conditions

Flies were raised on Lewis medium [37]. All experiments were conducted at 25ºC on a 12 hr:12 hr light:dark cycle.

Feeding Assays

Flies were collected under brief (<2 min) CO^2 anesthesia. Mature (4-day-old) virgin females were divided in two groups, "virgins" (20 females/vial) and "mated" (15 females + 5 males/vial) and kept for 3 days, with fresh food being provided on the second day. Assays were conducted immediately after the mating period. In the case of females expressing SP, and thus exhibiting enhanced rejection behavior, the flies were kept as "virgins" (20 females/vial) or "mated" (10 females + 10 males per vial). All the above conditions consistently resulted in the insemination of 100% of females in the "mated" group.

The dye assay was performed as follows: For visualization (Figure 1A), ad-libitum-fed flies were allowed to feed on red-colored (FD & C Red 40) Lewis medium for 2 hr, anesthetized, and imaged. For quantitation (Figure 1B), flies were allowed to feed on colored medium for 30 min following 12 hr of starvation in vials containing moist filter paper. Abdomens were isolated and homogenized in 1 × Phosphate Buffer Saline, and OD was recorded at 630 nm in a Benchmark Plus microplate spectrophotometer (BioRad).

The radioactive assay was carried out essentially as described [18]: Flies were allowed to feed for 24 hr on medium containing 0.35 nCi/μL [α-^{32}P]dCTP (MP Biomedicals), switched to empty shell vials and allowed to groom for 30 min, anesthetized by cold, transferred to scintillation vials, and covered with 10 mL scintillation fluid (Research Products International). Scintillation was recorded with a Beckman LS 5000 TA Liquid Scintillation System. Background signal (defined as scintillation counts recorded from a sample fed nonradioactive food) was subtracted from raw values. Each trial included two [α-^{32}P]dCTP calibration samples, which were used to convert scintillation counts to ingestion volume.

Statistics

Graphpad Prism software package was utilized for all statistical analyses.

Supplemental Data

Supplemental Data include two figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/7/692/DC1/.

Acknowledgments

We thank members of the Benzer lab, E. Kubli, and T. Aigaki for helpful discussions. This work was supported by a Lawrence L. and Audrey W. Ferguson Fellowship to G.B.C., a grant from the American Federation for Aging Research and a postdoctoral fellowship from the John Douglas French Foundation for Alzheimer's Research to P.K., and grants to S.B. from the National Institutes of Health (AG16630, AG24366, and DK070154), the National Science Foundation (MCB-0418479), and the Ellison foundation.

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Supplemental Data

Allocrine Modulation of Feeding Behavior by the Sex Peptide of *Drosophila*

Gil B. Carvalho, Pankaj Kapahi, David J. Anderson, and Seymour Benzer

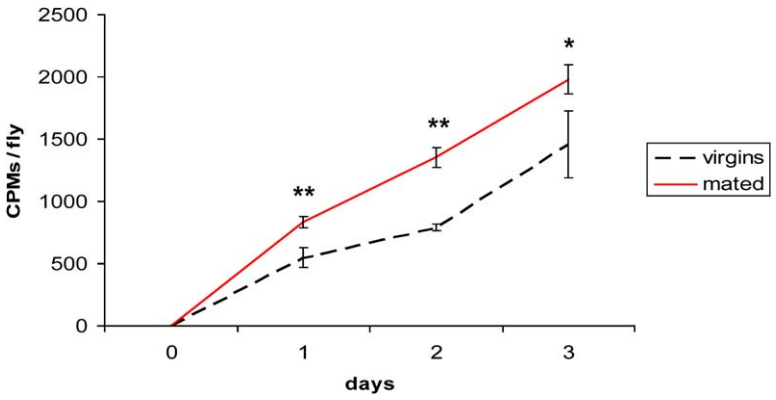

Figure S1. Dynamics of Radioactive-Signal Accumulation

Signal intensity was recorded every 24 hr over a total period of 72 hr on radioactively labeled food medium. Slope = 19.3 ± 2.6 (virgins) and 28.2 ± 1.8 (mated) (nonlinear regression analysis, 95% confidence interval). CPMs denotes counts per minute. Error bars represent standard deviation (SD) of three replicates per condition per time point. * indicates $p < 0.05$; ** indicates $p < 0.01$, two-tailed t test.

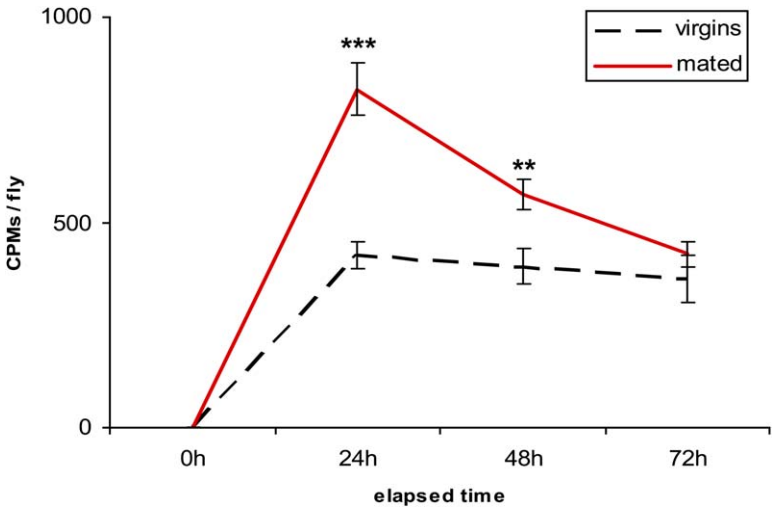

Figure S2. Pulse-Chase of Radioactive-Signal Incorporation

Flies were allowed to feed on isotope-containing medium for 24 hr and were subsequently transferred to nonlabeled medium of identical composition. Signal intensity was recorded immediately before, and 24 and 48 hr after, the transfer. CPMs = counts per minute. Error bars represent SD of four replicates per condition per time point. ** indicates $p < 0.01$; *** indicates $p < 0.0001$, two-tailed t test.

Chapter 3

Prandiology of *Drosophila* and the CAFE Assay

This chapter consists of a paper coauthored by William W. Ja, Elizabeth M. Mak, Noelle N. de la Rosa, Annie Y. Fang, Jonathan C. Liong, Ted Brummel and Seymour Benzer, which appeared in *Proceedings of the National Academy of Sciences*. Here, we describe the Capillary Feeder (CAFE), a method allowing direct, real-time measurement of feeding rate in *Drosophila*. The CAFE was developed to circumvent the caveats of isotope labeling. The two methods are complementary. Together, they allow a thorough qualitative and quantitative characterization of *Drosophila* feeding behavior.

Prandiology of *Drosophila* and the CAFE assay

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Contributed by Seymour Benzer, March 26, 2007 (sent for review March 14, 2007)

Studies of feeding behavior in genetically tractable invertebrate model systems have been limited by the lack of proper methodology. We introduce the Capillary Feeder (CAFE), a method allowing precise, real-time measurement of ingestion by individual or grouped fruit flies on the scale of minutes to days. Using this technique, we conducted the first quantitative analysis of prandial behavior in *Drosophila melanogaster*. Our results allow the dissection of feeding into discrete bouts of ingestion, defining two separate parameters, meal volume and frequency, that can be uncoupled and thus are likely to be independently regulated. In addition, our long-term measurements show that flies can ingest as much as 1.7× their body mass over 24 h. Besides the study of appetite, the CAFE can be used to monitor oral drug delivery. As an illustration, we used the CAFE to test the effects of dietary supplementation with two compounds, paraquat and ethanol, on food ingestion and preference. Paraquat, a prooxidant widely used in stress tests, had a strong anorexigenic effect. In contrast, in a feeding preference assay, ethanol-laced food, but not ethanol by itself, acted as an attractant.

appetite | feeding | ingestion | preference

Understanding the physiology and regulation of appetite is an indispensable step in tackling biomedical problems such as obesity and feeding disorders. Invertebrate model systems have provided invaluable mechanistic insight into the genetic control of various biological and pathological processes, but have contributed relatively little to the understanding of the genetic underpinnings and neuronal circuitry of appetite regulation. This dearth is largely due to the limits of the available methodology. In both *Caenorhabditis elegans* and *Drosophila melanogaster*, feeding behavior is often inferred from qualitative parameters such as the amount of time spent on a given food source or the percentage of animals from a population seen eating or simply loitering on the medium at a given time (1–3). A more direct method, widely used in the nematode, is the pharyngeal pumping rate, which assumes a constant ingestion volume per pharyngeal contraction (4–6). In *Drosophila*, food can be labeled with nonabsorbable dyes (6, 7) or radioactive isotopes (8–12), but these techniques also have several limitations. Dyes progress rapidly through the digestive tract, precluding long-term measurements. Isotope labeling, on the other hand, permits long-term recordings but does not distinguish between ingestion and intestinal absorption, leading to permanent tissue incorporation. Most importantly, labeling methods require killing the flies for each measurement, making it impossible to continuously monitor the behavior of individual animals.

We describe a method allowing unambiguous recording of food ingestion in individual or groups of flies on the scale of minutes to the entire lifespan. Monitoring ingestion at short, 10-min intervals permitted the delineation of single meals. By modulating nutrient composition, we show that the parameters of meal volume and frequency are under independent control. In addition, we illustrate the usefulness of the Capillary Feeder (CAFE) for drug delivery.

Results and Discussion

Inspired by the work of Dethier with the blowfly *Phormia regina* (13, 14), we developed the CAFE, an assay allowing precise, continuous quantitation of actual ingestion in individual *Drosophila*. In the CAFE, flies consume liquid food from a graduated glass microcapillary (Fig. 1). Descent of the meniscus is clearly visible, allowing continuous, unambiguous measurement of consumption. This method obviates the need for food markers and the commonly used supportive ingredients, such as cornmeal and agar. Because the capillaries can be replaced as needed, with minimal disturbance to the animals, it is possible to monitor real-time ingestion for periods ranging from minutes to the entire lifespan.

Although much attention has been devoted to the analysis of appetite, most studies have focused on total ingestion. Prandiology, the study of specific parameters such as the size and frequency of meals, has been neglected, despite the central role played by prandial habits in the physiopathology of obesity, hypercholesterolemia, and heart disease (15). Because the sensitivity of the CAFE makes it possible to monitor ingestion on the scale of minutes, we studied the short-term feeding pattern of individual flies. This analysis revealed discrete feeding events (meals) separated by intervals of no consumption (Fig. 2*A*). With a regimen of 5% sucrose + 5% autolyzed yeast extract, we recorded an average meal volume of 0.096 ± 0.008 μl at a frequency of 0.65 ± 0.08 meal/h (Fig. 2*C*).

We next asked whether meal size and frequency can be uncoupled by manipulating food composition. Male flies feeding on a 5% sucrose solution, with no yeast extract added, showed a meal frequency similar to that of flies fed sucrose + yeast (average = 0.58 ± 0.14 meal per h, $P = 0.64$) (Fig. 2 *B* and *C*). In contrast, average meal volume increased by 56% (0.15 ± 0.02 μl, $P < 0.003$) (Fig. 2*C*). Hence, *Drosophila* feeding behavior is a function of at least two discrete, independently regulated components. It should therefore be possible to isolate mutants affecting each feeding parameter separately.

The CAFE can be used to record ingestion continuously over an extended period. We monitored individually housed male flies fed 5% sucrose + 5% autolyzed yeast extract over a 5-day period (Fig. 3*A*). Flies consumed a daily average of 1.5 ± 0.04 μl, an impressive 1.7× their body mass. This value varied between 1.3 and 2.3 μl per day per fly in different experiments. The rate of ingestion varies during the 12-h light/dark periods (data not shown). Approximately two-thirds of the daily total ingestion occurs during the light period. The linearity of the long-term accumulation patterns in Fig. 3 is due to the individual measurements being made twice daily, once during the mid-light and the other during the middark periods, and therefore does not reveal the circadian rhythm.

In *Drosophila*, social interaction can influence courtship, aggression behavior, and sleep patterns (16, 17). We compared the ingestion by flies housed individually, in pairs, or in groups

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The authors declare no conflict of interest.

Abbreviation: CAFE, Capillary Feeder.

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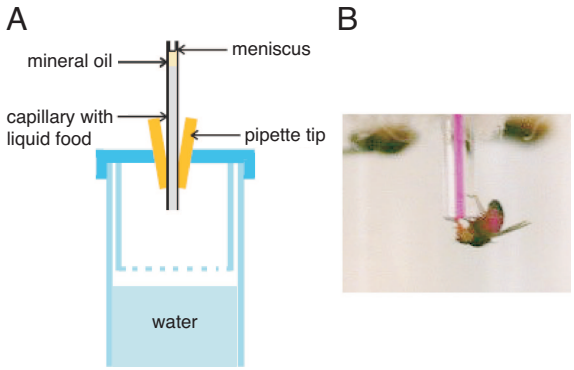

Fig. 1. The CAFE assay. (*A*) Schematic diagram. Liquid food, topped with an oil layer to minimize evaporation, is introduced via a glass capillary held in place by a pipette tip. The pierced bottom of the inner chamber provides humidity. (*B*) Fly feeding from the capillary. To facilitate visualization, a red dye has been added to the medium and can be seen in the proboscis and abdomen of the fly.

of four or eight animals per CAFE. Average ingestion per fly was identical in all groups (Fig. 3*B*), suggesting that, under the conditions used, food consumption in the CAFE is not significantly influenced by the presence of conspecifics or competition for food access. When three flies were housed per chamber, changing the number of capillaries between one and three did not influence total feeding (Fig. 3*C*), supporting the conclusion that, under these experimental conditions, the amount of and access to the food source are not limiting.

To feed in the CAFE, flies must climb onto the glass capillary and descend to reach the tip (Fig. 1*B*). Access to the medium can therefore be more strenuous than that under ordinary laboratory conditions, where flies stand on abundant solid food. We asked whether ease-of-access to the nutrient source influences ingestion volume by varying the distance between the capillary tip and the top of the chamber, on which the flies tend to accumulate and wander. For one group, the capillary opening was set immediately below the pipette tip, i.e., 4 mm below the cap (Fig. 1*A*), allowing the flies to feed without having to climb down on the capillary. In a second group, the tip was placed 6.5 mm below the cap (the default condition used in all other experiments reported here), whereas a third group had the tip placed 16.5 mm below. These variations in capillary height had no effect on ingestion rates (Fig. 3*D*). Under all conditions tested, the flies were never observed to jump or fly directly onto the capillary, instead choosing to walk from the cap onto the glass surface and treading its length to reach the opening. The conditions of the CAFE are therefore unlikely to inhibit feeding by reducing food accessibility.

Pharmacological treatments are a hallmark of behavioral and metabolic studies in *Drosophila* (18–20). The CAFE represents a significant advance for oral drug delivery, because it minimizes the amount of material required, while confirming actual ingestion and monitoring possible effects of the drug on appetite. To illustrate this application, we tested the effect of paraquat, a prooxidant drug commonly used in stress resistance tests. We compared the intake of animals offered a 5% sucrose solution with or without 20 mM paraquat. Over a 12-h period, the flies fed paraquat-laced food consumed 75% less than controls (0.23 ± 0.06 and 0.88 ± 0.16 μl, with or without paraquat; $P = 0.01$) (Fig. 4*A*). Moreover, monitoring prandial behavior during the first 6 h showed a decrease in average meal size from 0.15 ± 0.02 to 0.05 ± 0.02 μl with paraquat ($P = 0.007$) (Fig. 4*B*). Throughout the 12-h period, flies retained their climbing ability, and paraquat-induced death did not begin until 36 h (data not shown). The observed difference in intake thus suggests a bona fide anorexigenic effect of the compound, rather than nonspecific morbidity. These results stress the importance of taking into account actual ingestion upon oral administration of drugs, which are typically added to solid food.

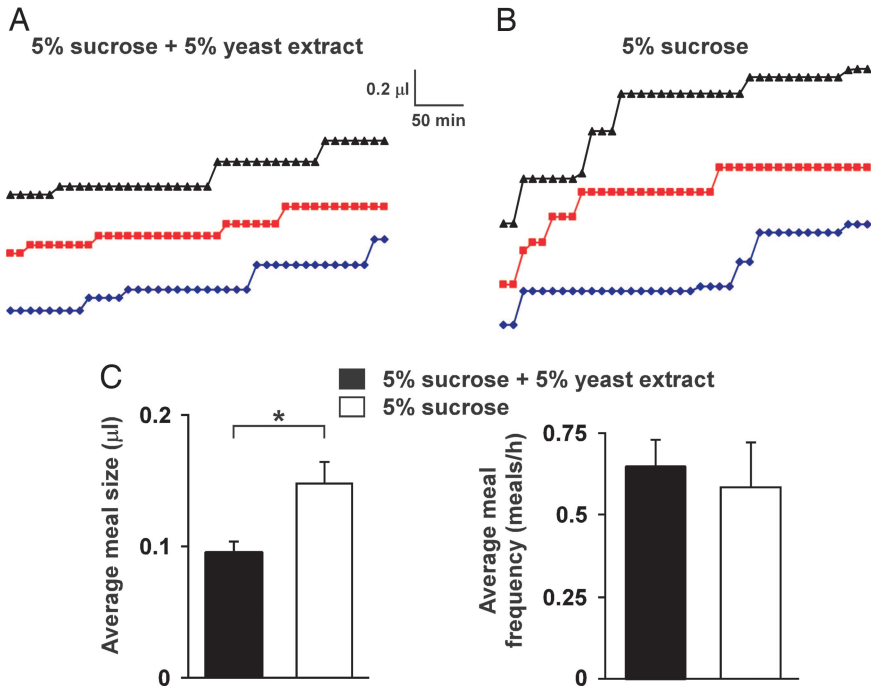

Fig. 2. Prandial behavior analyzed in the CAFE. (*A*) Intake by three individually housed male flies fed 5% sucrose + 5% yeast extract measured in 10-min intervals. A vertical rise flanked by two intervals of no intake was defined as a meal. (*B*) Intake by individual flies fed 5% sucrose. (*C*) Meal volume and frequency can be decoupled by modulating nutrient conditions. On 5% sucrose, average meal size increases, whereas meal frequency is unchanged; 5% sucrose + 5% yeast extract: $n = 10$ flies, 52 meals; 5% sucrose: $n = 4$ flies, 18 meals. All values are given as averages ± SE. *, $P \leq 0.01$, two-tailed *t* test.

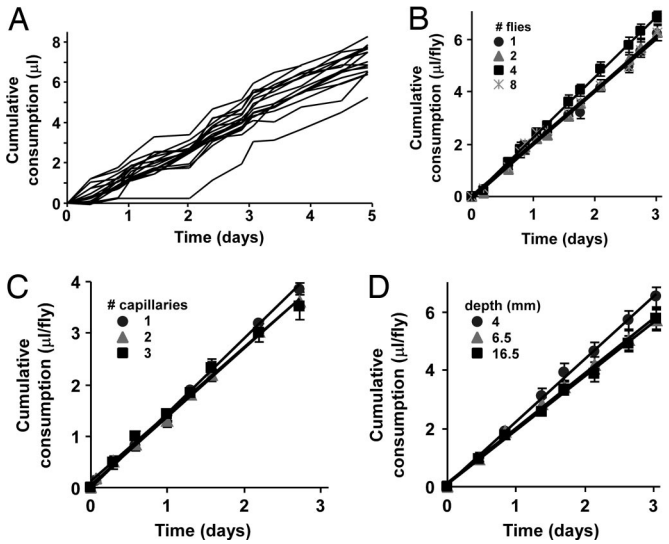

Fig. 3. Measurement of long-term food consumption in the CAFE. (*A*) Cumulative ingestion by 17 individual male flies over 5 days. Average consumption = 1.5 ± 0.04 μl per day per fly. (*B*) The number of animals per chamber does not influence individual feeding rate. One, two, four, or eight flies were housed per CAFE. Average consumption was 2.0 ± 0.02, 2.1 ± 0.1, 2.3 ± 0.1, and 2.0 ± 0.1 μl per day per fly, respectively ($R^2 > 0.98$ for each linear fit; ANOVA $P = 0.24$). (*C*) The number of capillaries per chamber does not affect food intake. One, two, or three capillaries were used per CAFE. Three flies were housed per chamber. Average consumption was 1.5 ± 0.03, 1.3 ± 0.04, and 1.3 ± 0.1 μl per day per fly, respectively ($R^2 > 0.98$; ANOVA $P = 0.25$). (*D*) Capillary depth has no effect on food ingestion. Four flies were used per CAFE, with the capillary tip placed 4 mm, 6.5 mm, or 16.5 mm below the top of the chamber, respectively (Fig. 1*A*). Average consumption was 2.2 ± 0.2, 1.9 ± 0.1, and 1.8 ± 0.1 μl per day per fly, respectively ($R^2 > 0.99$; ANOVA $P = 0.26$). In all experiments, 5% sucrose + 5% autolyzed yeast extract was served. All values are given as averages ± SE.

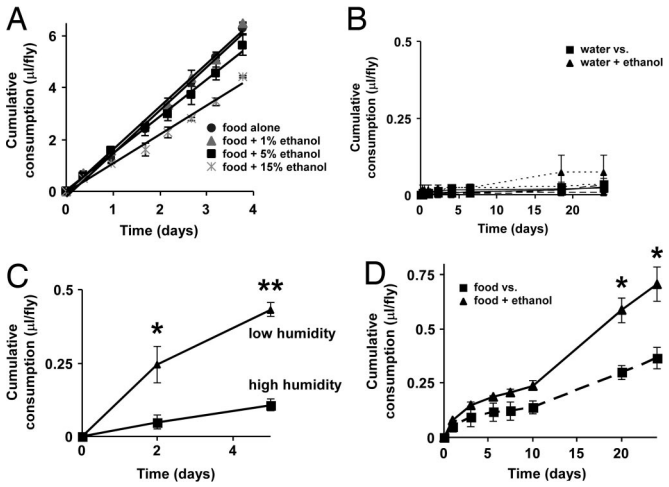

Fig. 5. Serving ethanol in the CAFE. (*A*) A dietary ethanol supplement has a modest, inhibitory effect on long-term food intake. Flies were fed 5% sucrose + 5% autolyzed yeast extract medium alone or supplemented with 1%, 5%, or 15% (vol/vol) ethanol. Average consumption was 1.7 ± 0.07, 1.7 ± 0.03, 1.4 ± 0.1, and 1.1 ± 0.01 μl per day per fly, respectively ($R^2 > 0.97$ for each linear fit; ANOVA $P = 0.018$; $n = 8$ flies per condition). (*B*) In the absence of food medium, ingestion of either plain water or ethanol is remarkably low. For 24 h, flies were offered a choice between two capillaries, one containing pure water and the other containing one of three concentrations of ethanol: 1% (dotted line), 10% (dashed line), or 50% (solid line). Maximum ingestion was <0.07 μl per day per fly with 1% ethanol ($n = 12$ animals per condition). (*C*) Desiccation stimulates water consumption. Flies were deprived of food and water for 24 h in either a humidified or nonhumidified CAFE and then provided with plain water in regular humidified conditions. (*D*) Given a choice between food (5% sucrose + 5% autolyzed yeast extract) with and without a 15% ethanol supplement, flies showed a strong preference for the ethanol-laced regimen ($n = 8$ animals per condition). All values are given as averages ± SE. *, $P < 0.05$; **, $P < 0.01$, two-tailed *t* test.

Alcoholism is a notorious health problem with major social and economic consequences. Epidemiological data indicate that 13.5% of the population in the United States suffers from alcohol abuse dependence (21). Elucidating the mechanisms of alcohol intoxication and addiction are, therefore, outstanding biomedical goals. In recent years, *Drosophila* has become a prominent model system for the study of drug physiology, with a significant number of studies centering on the effects of ethanol (18, 22, 23). However, most studies have relied on ethanol vapor, which may bear differences from oral ingestion. The CAFE readily lends itself to studies of feeding facilitation. We therefore set out to develop a protocol for oral administration of ethanol by using the CAFE. We continuously monitored the consumption of 5% sucrose + 5% autolyzed yeast extract supplemented with various concentrations of ethanol over 4 days. A 1% supplement had no effect on feeding, but adding 5 or 15% ethanol resulted, respectively, in 14% and 33% lower overall consumption (Fig. 5*A*). This effect seems relatively modest in light of the high caloric content of ethanol: The presence of 5% and 15% ethanol, respectively, doubles and quadruples the total caloric value of the medium (medium alone = 279 kcal/liter, medium + 5% ethanol = 555 kcal/liter, and medium + 15% ethanol = 1,107 kcal/liter; see *Materials and Methods*). This finding may suggest that flies only absorb and/or metabolize a fraction of the ethanol they ingest. Alternatively, caloric content may not be the main determinant of feeding rate in *Drosophila*. In any case, our work establishes a method for oral administration of ethanol to *Drosophila* over extended periods of time.

We next asked whether ethanol represents an attractive or aversive stimulus when presented acutely. In the absence of food, flies ingested a negligible amount of ethanol in any of three concentrations [1%, 10%, or 50% (vol/vol)], even when housed in the CAFE up to 24 h and therefore under considerable nutrient deprivation (Fig. 5*B*). Similarly, pure water was ingested in remarkably small amounts over the same period (Fig. 5*B*). This is attributable to the high humidity maintained in the chambers because flies starved in a nonhumidified CAFE (with no water in the outer chamber) showed significantly increased ingestion of pure water from the capillaries (Fig. 5*C*). These results demonstrate that ethanol alone does not represent a particularly attractive stimulus. Together with the long-term

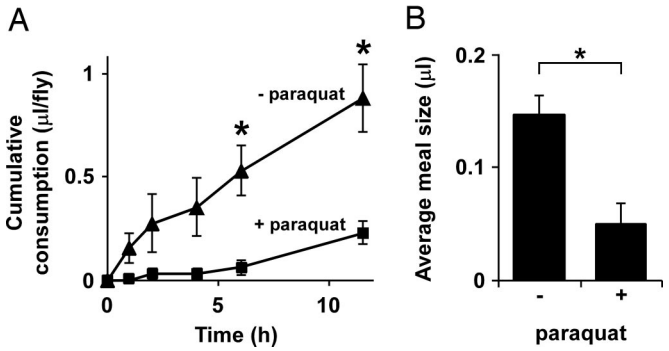

Fig. 4. Dietary paraquat inhibits food intake. (*A*) Ingestion of a 5% sucrose solution with or without 20 mM paraquat over a 12-h period ($n = 5$ flies per condition. (*B*) Paraquat inhibits meal size. Consumption was recorded every 10 min during the first 6 h of the long-term experiment shown in *A*. All values are given as averages ± SE. *, $P < 0.01$, two-tailed *t* test.

results (Fig. 5*A*), which suggest that it is not particularly aversive either, this result raised the possibility that flies are unable to detect ethanol. As a more stringent test of this scenario and of the valence of this substance, we conducted a feeding preference test in which flies were offered a choice between medium with or without a 15% ethanol supplement in two separate capillaries. Surprisingly, this test revealed a clear preference for the alcohol-containing meal (Fig. 5*D*). Together, our results indicate that ethanol constitutes an attractive stimulus in the presence of food but not by itself. A possible explanation is that ethanol itself possesses an indifferent taste but confers a metabolic advantage, such as a concentrated source of calories. Flies therefore do not ingest it when presented in isolation, but upon sampling it in their food associate that particular meal with the acquired metabolic advantage. Alternatively, the specific combination of ethanol and food may represent an attractive gustatory stimulus. More work will be required to distinguish between these possibilities and elucidate the mechanism of the ethanol preference behavior.

We have shown that the CAFE assay can reliably measure short- and long-term food ingestion of individual or groups of flies, as well as identify both inhibitory and stimulatory effects of dietary compounds on appetite. Because the CAFE requires orders of magnitude less material than the addition of drugs to solid food and because it allows simultaneous monitoring of intake, it will represent a significant advance for drug screens, in which the quantity of reagents can be a limiting factor. The CAFE should be of great value for the analysis of genetic pathways and neuronal circuits that regulate appetite in *Drosophila*, such as the insulin-like signaling pathway, hugin, and neuropeptide F (24, 25). Additionally, it should be adaptable to an automated, multi-CAFE, high-throughput format.

Materials and Methods

Preparation of the CAFE. The model used for these experiments was composed of two chambers (Fig. 1). The inner chamber, containing the flies, was prepared by paring down a 1.5-cm diameter plastic vial to 2-cm length, with the bottom pierced to allow entry of water vapor and air from the outer chamber, a 50-ml conical tube filled with 30 ml of water. Calibrated glass micropipettes (5 μl, catalog no. 53432-706; VWR, West Chester, PA) filled with liquid medium by capillary action were inserted through the cap via truncated 200-μl pipette tips. For some experiments, a mineral oil overlay ($\approx$0.1 μl) was used to minimize evaporation. Capillaries were replaced as needed. The long-term experiment in Fig. 3*A* was conducted under a 12-h-light/12-h-dark cycle in a room kept at 25°C and >70% humidity. The prandiology studies of Fig. 2 were conducted during the light period. The choice experiments in Fig. 5 were performed with two labeled capillaries, each containing a different food. Each experiment included an identical CAFE chamber without flies to determine evaporative losses (typically <10% of ingested volumes), which were subtracted from experimental readings. Average values ± SE are given.

Flies and Media. All flies tested were $\approx$1-week-old males of the Canton Special (Canton-S) strain raised on the Lewis medium used at the California Institute of Technology (26) and transferred to the CAFE from this food. Except where otherwise specified, the liquid food used in the CAFE was 5% (wt/vol) sucrose + 5% (wt/vol) autolyzed yeast extract (Bacto yeast extract; BD Diagnostic Systems, Franklin Lakes, NJ). All flies were habituated in the CAFE for 24 h, with ad libitum medium, before the measurements were started. The caloric content of the medium was calculated on the basis of the following values: 4 kcal/g (sucrose), 1.58 kcal/g (yeast extract), and 7 kcal/g (ethanol).

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Chapter 4

Water- and Nutrient-Dependent Effects of Dietary Restriction on *Drosophila* Lifespan

This chapter consists of a paper coauthored by William W. Ja, Brian M. Zid, Elizabeth M. Mak, Ted Brummel and Seymour Benzer, which appeared in *Proceedings of the National Academy of Sciences*. The work shows that, in several common experimental paradigms, *ad libitum* water availability eliminates the lifespan-extending effect of food dilution. We also characterize a paradigm of DR that extends lifespan irrespective of water availability. The simplest explanation for these findings is that the field of *Drosophila* DR has studied an experimental artifact – dehydration of flies provided concentrated media –, rather than an effect of differential nutrient ingestion, as commonly assumed. The main implication of this work is that the phenomenon classically studied in flies is likely different from mammalian DR, since the latter impacts lifespan in the presence of *ad libitum* water. In conclusion, this paper casts serious doubt on the relevance of most of the work done previously in the fly DR field and proposes a new paradigm that likely represents a more meaningful model for mammalian DR.

Water- and nutrient-dependent effects of dietary restriction on *Drosophila* lifespan

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Dietary restriction (DR) is a widely conserved intervention leading to lifespan extension. Despite considerable effort, the mechanisms underlying DR remain poorly understood. In particular, it remains unclear whether DR prolongs life through conserved mechanisms in different species. Here, we show that, in the most common experimental conditions, lifespan extension by DR is abolished by providing *Drosophila* with ad libitum water, without altering food intake, indicating that DR, as conventionally studied in flies, is fundamentally different from the phenomenon studied in mammals. We characterize an alternative dietary paradigm that elicits robust lifespan extension irrespective of water availability, and thus likely represents a more relevant model for mammalian DR. Our results support the view that protein:carbohydrate ratio is the main dietary determinant of fly lifespan. These findings have broad implications for the study of lifespan and nutrition.

aging | caloric restriction | dehydration | longevity | nutrition

Dietary restriction (DR), classically defined as a reduction in nutrient availability short of malnutrition, can extend the lifespan of organisms ranging from yeast to mice (1, 2). In rodents and primates, DR delays the onset of age-related pathologies, such as cancer, cardiovascular disease, and diabetes (3–5). Chronic DR also elicits a number of physiological changes, including decreased circulating glucose, insulin, and cholesterol levels; reduced body mass; and compromised reproductive function (5–8). Despite the evident biomedical interest in DR, its mechanistic basis remains largely unknown, and it is unclear whether DR extends lifespan in different species through similar mechanisms (9, 10). This issue is of fundamental importance, since invertebrate model systems are especially valued for their ability to provide mechanistic clues to be tested in mammals.

In mammals, food restriction is imposed by feeding the DR cohort a fraction of that ingested by the ad libitum group (2). Due to the difficulty of controlling feeding rates in invertebrates, more ingenious, albeit potentially problematic, techniques are used. *Drosophila* DR is commonly achieved by total food dilution (11) and carried out in the absence of a separate water source, unlike with other species (12–16). Hence, fly food is simultaneously the source of nutrients* and water. This setup prevents flies from independently regulating nutrient and water intake, leaving room for the possibility that any effects of food dilution are mediated by changes in hydration.

Our results show that lifespan extension by typical DR regimes (17–20) can be entirely abolished by providing flies with free access to water. Water supplementation does not affect food consumption, suggesting that DR, as typically applied, does not impact longevity through reduced nutrient intake. Furthermore, we characterize a regime that elicits robust lifespan extension independent of water supplementation. Our findings suggest that most of the work done on *Drosophila* DR has been confounded by changes in hydration. In conditions where water intake is not limiting, lifespan modulation by DR can be explained by the protein:carbohydrate ratio.

Results and Discussion

We measured the water consumption of flies fed two different concentrations (dietary restriction, DR; concentrated medium, CM) of yeast extract/sucrose (YE/S) medium by providing ad libitum water labeled with a radioactive tracer (21–23). Flies were housed in population cages containing separate food and water sources of similar surface area. CM-fed flies drank five times as much as those on DR, and this trend was maintained on an even richer medium (Fig. 1*A*). The difference in water content of the food (CM = 0.86 ± 0.04 and DR = 0.98 ± 0.06 mL H_2O/mL medium, respectively) seems mild compared with the dramatic difference in water ingestion. We reasoned that compensatory feeding, the ability of flies to regulate their intake in response to changes in food concentration (24), might play a causal role. Thus, animals that restrict their intake to compensate for the high concentration of CM would consequently ingest less liquid and require an independent water source. Food dilution indeed had a strong phagostimulatory effect (Fig. 1*B*). Isotope accumulation was near linear for several days (Fig. S1*A*), and flies fed diluted food were not less efficient in eliminating or metabolizing the label (Fig. S1*B*), supporting the validity of radioactivity measurements (25, 26). Notably, food intake was unaffected by water access (Fig. 1*B*). These results were independently confirmed using the Capillary Feeder (CAFE) assay, which directly measures consumption (Fig. S1*C*) (27). Similar results were observed with both genders, as well as with different media, fly strains, and enclosures (Fig. 1 *C*–*F*). These experiments span the most common paradigms of *Drosophila* DR.

Collectively, our results indicate that, faced with the common food/water sources used in DR, flies give priority to regulating their nutrient intake via compensatory feeding, at the expense of optimal hydration. Since flies exposed to richer media are significantly thirstier than controls (Fig. 1 *A*, *C*, and *E*), we asked if this state of chronic dehydration affects longevity. Lifespan was measured on DR and CM with and without water supplementation. In the control group, the aqueous medium (1% agar) was covered by a nylon mesh, preventing access to the water source while ensuring identical humidity. Strikingly, ad libitum water access prolonged the survival of CM-fed flies to the level of their DR cohorts, whereas the latter experienced only a mild benefit in the presence of water (Fig. 2 *A* and *B*). As a result, DR extended lifespan in the absence, but not in the presence, of the aqueous source (Fig. 2*C* and Table S1). The nonadditive effect of DR and water supplementation on lifespan is not due to an

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The authors declare no conflict of interest.

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*Although water is commonly considered a nutrient, we use this term to refer to all nonwater food components (e.g., yeast and sugar) throughout the text.

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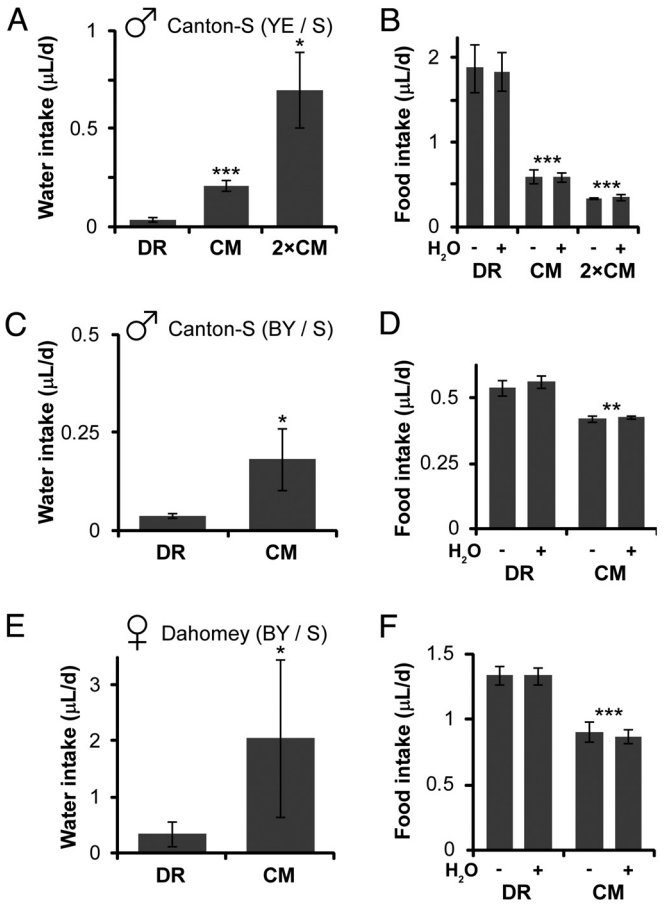

Fig. 1. Food and water intake assayed by medium radiolabeling (22). (*A* and *B*) Yeast extract/sucrose (YE/S)-fed Canton-S males maintained in demography cages. (*C* and *D*) Brewer's yeast/sucrose (BY/S)-fed Canton-S males maintained in demography cages. (*E* and *F*) BY/S-fed Dahomey females maintained in vials. Flies drink greater volumes of water and consume less of the food as concentrations of YE/S (*A* and *B*) or BY/S (*C*–*F*) increase. Results are expressed as an average (± SD) of 2–6 trials, each containing 6–16 flies. Food composition, YE/S: *DR* = 2.5% YE + 2.5% S; *CM* = 10% YE + 10% S; 2×*CM* = 20% YE + 20% S; BY/S: *DR* = 10% BY + 5% S; *CM* = 20% BY + 10% S (all wt/vol). Statistical significance was determined by nonpaired, two-tailed Student's *t* tests between results on DR and CM or CM and 2×CM media: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (*B*, *D*, and *F*) The presence of water did not affect food intake on any medium ($P > 0.05$).

absolute lifespan "ceiling" or maximum, since other conditions imparted greater longevity (Table S2).

We tested the effect of water access on lifespan in different genders, fly strains, media, and animal enclosures. Water supplementation mimicked the longevity-enhancing effect of DR in all conditions tested (Fig. 2 *D*–*I*, and Tables S1 and S2), with no significant effect on food intake (Fig. 1 *B*, *D*, and *F*). The magnitude of lifespan extension by DR in the absence of water is in agreement with reported results (17). Notably, one of our experiments (Fig. 2 *G*–*I*) faithfully replicated the conditions recently proposed as the most appropriate for DR experiments on the basis of their effects on lifespan and fecundity (17). The simplest explanation for these findings is that the typical conditions used in *Drosophila* DR and water supplementation extend lifespan through largely overlapping mechanisms.

Our findings contradict a recent study that found no effect on lifespan when water was provided in a pipette tip, although water consumption was not confirmed (17). The substantially larger water surface used here may enhance access or counter crowding effects, microorganism growth, accumulation of excreta, or other undetermined factors.

Although DR has been established on yeast extract/sucrose/cornmeal (YE/S/C) upon varying yeast alone (28, 29), this paradigm is much less commonly used. In contrast to all other regimes tested, YE/S/C elicited negligible water consumption both in its low- and high-yeast forms (Fig. S2*A*), indicating that YE/S/C is unique in its ability to satisfy the animals' water needs. Interestingly, reducing yeast levels in YE/S/C stimulated food ingestion (Fig. S2*B*), demonstrating that compensatory feeding does not necessarily result in dehydration, and suggesting that water needs are determined by an interplay between feeding behavior and the specific nutrient source.

In the YE/S/C paradigm, DR prolonged lifespan in males and females irrespective of water access (Fig. 3). The small increase in lifespan of CM-fed males with water access was not reproducible (Fig. S3). Importantly, female fecundity directly correlated with yeast levels (Fig. 4), arguing against a toxic effect of yeast extract (9). Hence, we have established a paradigm where nutrient manipulation has a clear impact on longevity irrespective of water availability.

The main goal of invertebrate research is to generate insights into the mechanisms of human biology, and thus an ideal fly DR model should bear analogy to the mammalian phenomenon. Since DR in mammals is generally conducted in the presence of ad libitum water (12, 13) and thus impacts longevity in hydrated animals, one would expect the fly paradigm to also be dependent on nutrient, rather than water ingestion. However, our findings show that the bulk of *Drosophila* DR studies (11, 17–20) have dissected a form of lifespan extension that is entirely dependent on water availability, and therefore differs fundamentally from the phenomenon studied in mammals. The YE/S/C water-independent paradigm more closely resembles mammalian DR and, therefore, likely represents a more relevant model of DR in higher organisms. Two observations lend further support to this view. First, maximum lifespan, arguably a better indicator of aging rate than average longevity (30), is robustly extended upon DR in rodents (4) and in the water-independent regime (Fig. 3 and Table S2), but not in the classical *Drosophila* DR paradigms (Fig. 2 and Table S2). Second, lifespan extension on YE/S/C was accompanied by a dramatic reduction in female fecundity that is reminiscent of the reproductive diapause seen in restricted rodents (31–33), whereas DR on water-dependent media has only a mild effect [Fig. 4 and (17)].

Our findings are consistent with the view that the protein-:carbohydrate (P:C) ratio is the main dietary determinant of fly longevity (34), a fact that may have been classically obscured by the hydration confound. As predicted by this model, classical DR paradigms, based on whole medium dilution and thus maintaining a constant P:C ratio, have only a mild, nutrient-independent effect on lifespan, whereas the YE/S/C paradigm used here, based on varying yeast alone and thus altering the P:C ratio, impacts longevity more dramatically, and in a nutrient-dependent manner. Notably, the low-yeast form of YE/S/C has a P:C ratio of 1:15, similar to the 1:16 described by Lee et al. as optimal for longevity (34). Other DR paradigms that alter the P:C ratio within the appropriate range without causing dehydration should be functionally equivalent to YE/S/C. Our results also demonstrate the remarkable plasticity of *Drosophila* feeding rate, in agreement with the finding that fly lifespan is determined by the interplay between P:C ratio and food intake (34). The lesson gleaned from these observations is that quantitative measurements of steady-state food intake are indispensable for any study aiming to understand the effects of nutrition on lifespan.

Our results directly contradict the long-held assumption that food manipulation affects fly lifespan solely through changes in

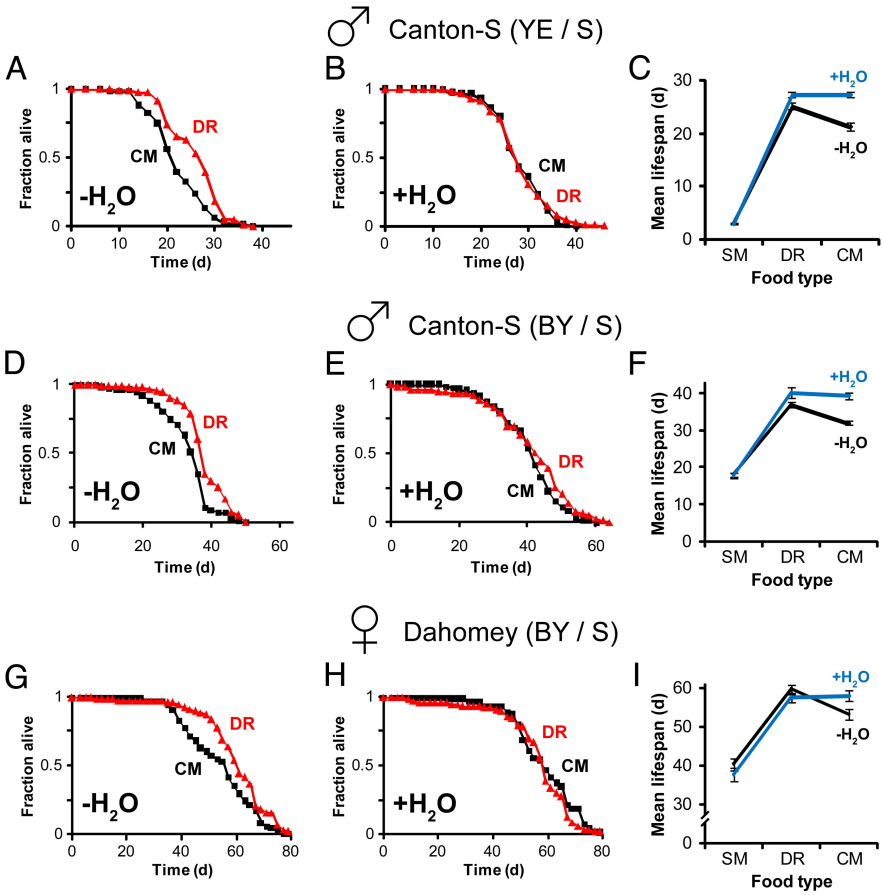

Fig. 2. Ad libitum water supplementation abolishes lifespan extension by dietary restriction (DR). (*A–C*) Yeast extract/sucrose (YE/S)-fed Canton-S males aged in demography cages. Lifespan curves without (*A*) and with (*B*) water supplementation of flies maintained on concentrated (CM) or DR medium. (*C*) Mean lifespan (± SEM) of flies on diets of CM, DR, or a heavily diluted starvation medium (SM) representing malnourishment. (*D–F*) Lifespan curves and mean lifespan, as in (*A–C*), respectively, of brewer's yeast/sucrose (BY/S)-fed Canton-S males aged in demography cages. (*G–I*) Lifespan curves and mean lifespan, as in (*A–C*), respectively, of BY/S-fed Dahomey females aged in vials. In the absence of a water source, the DR diet extended lifespan compared with flies fed CM in all conditions tested (*A*, $P = 1.3 \times 10^{-4}$; *D*, $P = 2.4 \times 10^{-6}$, *G*, $P = 2.5 \times 10^{-3}$; log-rank test). (*B*, *E*, and *H*) DR had no effect ($P > 0.05$, log-rank test) on lifespan upon water supplementation. Food composition is described in Fig. 1; YE/S: *SM* = 0.1% YE + 0.1% S; BY/S: *SM* = 1% BY + 0.5% S (all wt/vol). n = 68–156 flies per trial. Statistics of Cox proportional hazards analysis, demonstrating the greatly reduced effect of DR upon water supplementation, are shown in Table S1.

nutrient ingestion. Since this erroneous view has pervaded the field since its inception (11), our observations warrant a careful reexamination of the entire body of work of *Drosophila* DR. Any insights stemming from work on fruit flies (18–20, 24, 35, 36) are potentially confounded by changes in hydration and thus difficult to extrapolate to mammalian DR. This caveat extends to the numerous mutants shown to regulate fly DR (e.g., 37, 38). Extensive validation will be required to assess their value as clues to DR and aging in higher organisms. All future work should employ conditions in which ad libitum water is either present or shown not to affect lifespan.

Experimental Procedures

Reagents. Bacto™ agar and yeast extract were from BD Diagnostic Systems, sucrose from Mallinckrodt Baker, brewer's yeast from MP Biomedicals, and cornmeal (yellow) from Quaker Oats. *Drosophila* bottles (polypropylene, 8 oz. round bottom) and vials (polystyrene, 25 × 95 mm) were purchased from VWR International. For lifespan measurements in cages (large embryo collection cage, Genesee Scientific), food and water were supplied in compartmentalized dishes (four-section plates, Fisher Scientific).

Food Preparation. Food compositions are provided in the figure legends. Agar (0.5% if the food contained cornmeal, otherwise 1%, wt/vol) was heated with continuous stirring in ddH_2O (10–20% less than the desired final volume) on a hot plate. Upon boiling, food components were added and the heat reduced. After simmering with vigorous stirring for 2 min, food was removed from heat and the final volume adjusted with ddH_2O. After cooling to <65°C, a mixture of propionic and phosphoric acids [0.4 and 0.06% (vol/vol) final, respectively] was added and the food dispensed into either vials (2 mL) or two of the four compartments of segmented Petri dishes (7 mL). Ad libitum water was supplied as 1% agar, boiled and cooled to <65°C, and dispensed onto vial walls (400 μL) approximately 3 cm from the bottom. For cages, polypropylene caps from 50-mL conical tubes were filled with 1% agar (2.5 mL) and affixed with double-sided tape to the empty compartments of the Petri dishes. For both vials and cages, the surface area of the water source was approximately 75% of that of the food medium. In half of the cage experiments, the agar-containing caps were covered with nylon mesh to maintain humidity while preventing flies from accessing the water source.

Lifespan Analysis. Flies were raised in bottles containing Lewis medium (39). Groups of enclosed adults (0–3 days old) were transferred to fresh bottles and allowed to mate for 2 days. Males and females were then separated under CO_2 anesthesia and randomly allocated to different media (approximately 20 flies per vial or 120–150 flies per cage). All enclosures were maintained at 25°C in a controlled light (12/12-h light/dark cycle) and humidity (>70%) environment. Flies were scored for survival and provided with fresh medium every 2–3 days. Enclosures were placed randomly in the incubator, and positions were rotated after each transfer to minimize the effects of microclimate. Statistical significance of different survivorship curves was determined by log-rank test. Cox proportional hazards analysis was also used to generate a hazard ratio for each experiment in the presence or absence of water (Table S1). When the hazard ratio is close to 1, DR has little effect on survival.

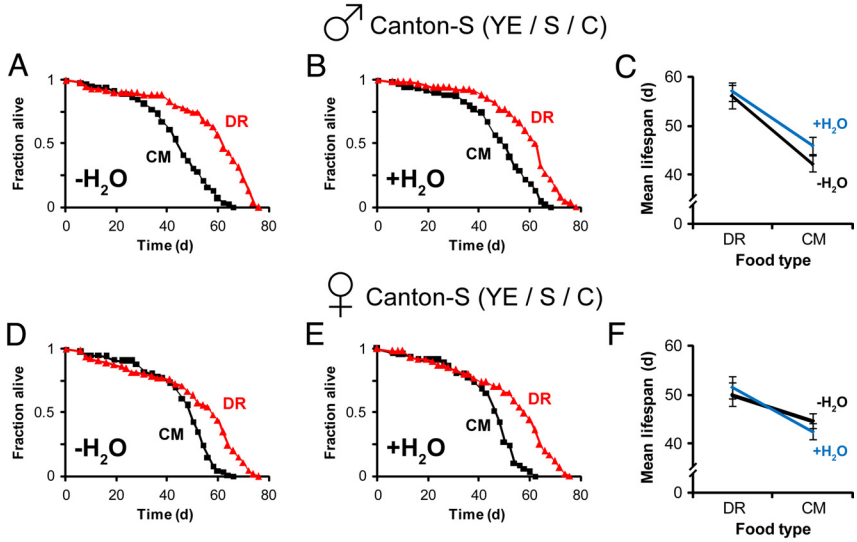

Fig. 3. Water supplementation does not affect lifespan extension upon reducing yeast alone on yeast extract/sucrose/cornmeal (YE/S/C). (*A–C*) YE/S/C-fed Canton-S males aged in demography cages. Lifespan curves without (*A*) and with (*B*) water supplementation of flies maintained on concentrated medium (CM) or dietary restriction (DR). (*C*) Mean lifespan (± SEM) of flies on CM or DR. (*D–F*) Lifespan curves and mean lifespan, as in (*A–C*), respectively, of YE/S/C-fed Canton-S females aged in demography cages. In this paradigm, reducing yeast extended lifespan in both the absence (*A* and *D*) and presence (*B* and *E*) of water ($P < 10^{-6}$, log-rank test). Food composition, YE/S/C: *DR* = 0.25% YE + 5% S + 8.6% C; *CM* = 5% YE + 5%S + 8.6% C (all wt/vol). n = 60–86 flies per trial. Statistics of Cox proportional hazards analysis are shown in Table S1.

Feeding Rate Measurement. Feeding assays were performed essentially as described (22). Briefly, adults (2–5 days old, approximately 10 flies/vial) were habituated for 4 days on the food medium being tested, with a transfer to fresh food on the second day. On day 4, flies were transferred to the same medium supplemented with 0.5–4 μCi/mL [α-^{32}P]-dCTP (<1.3 nM, final, MP Biomedicals), allowed to feed for 24 h, and then transferred to empty vials for 30 min. Cold-anesthetized flies were assayed in 10 mL of scintillation fluid (Research Products International) on an LS 5000 TA Liquid Scintillation System (Beckman Coulter). Flies fed nonlabeled food were used as blanks and the values were subtracted from experimental readings. Aliquots of the radioactive tracer were used to calculate food volumes from scintillation counts. Flies accumulated radioactive tracer at a near-linear rate for at least several days (Fig. S1 *A* and *B*).

Nutritional Information. Water content of the media was determined by preparing food as described above and dispensing 10 mL into preweighed containers. After the food had solidified, mass was measured and food density was calculated. Subtraction of the dry weight of the food components provided the water content. Protein and carbohydrate content of food components was taken from manufacturer's information (yeast extract: 51% protein + 16.33% carbohydrate; cornmeal: 7.5% protein + 78.8% carbohydrate, which include simple and complex carbohydrates).

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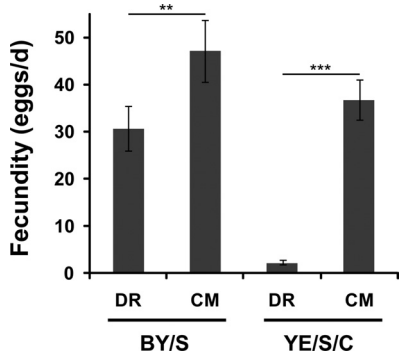

Fig. 4. Effect of food concentration on fecundity in different dietary paradigms. Increasing nutrient concentration in BY/S and YE/S/C diets stimulates the fecundity of Dahomey females. Results are comparable to the estimated lifetime fecundity on these foods (17). Mated Dahomey females (2–4 days old) maintained on the indicated medium were transferred daily and eggs counted for 12 days. Results represent the average number of eggs laid per day, per fly from days 2–12 and are expressed as the average (± SD) of 5 replicates containing 2 flies each. Medium composition is described in Figs. 1 and 3. Statistical significance was determined by nonpaired, two-tailed Student's *t* tests between DR and CM: **, $P < 0.01$; ***, $P < 0.001$.

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Supporting Information

Ja et al. 10.1073/pnas.0908016106

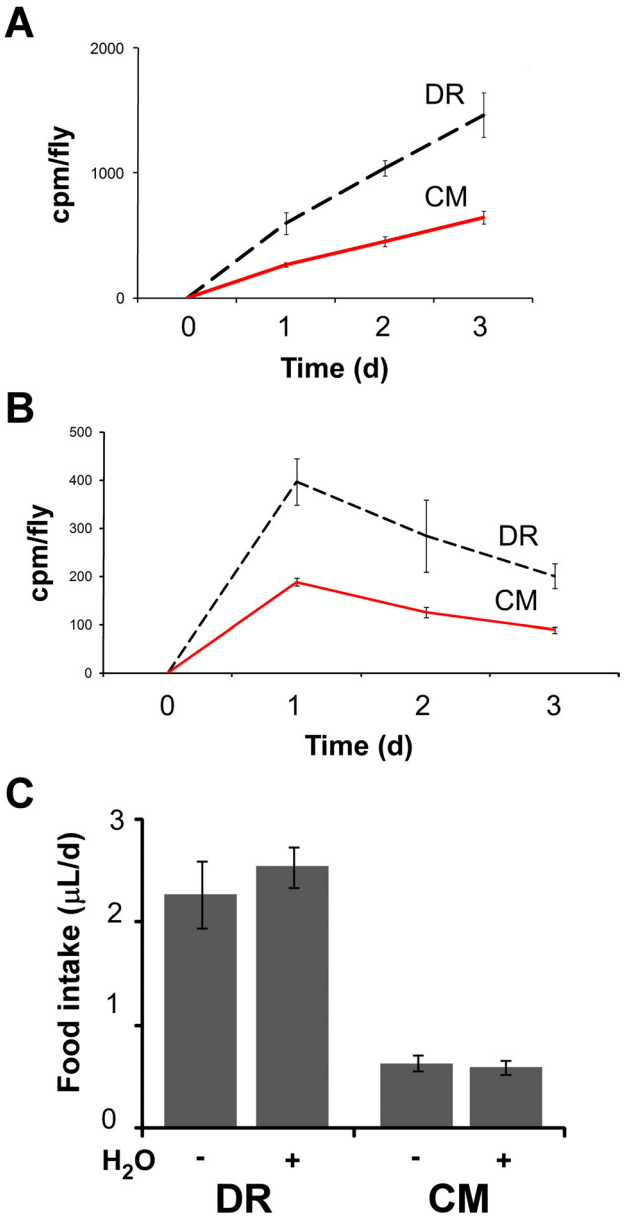

Fig. S1. Food dilution elicits compensatory feeding as measured by radiolabeling the medium (1–3) or by using the Capillary Feeder (CAFE) assay (4). (*A*) Time-course of isotope accumulation for flies fed yeast extract/sucrose (YE/S: CM = 5% YE + 5% S; DR = 1% YE + 1% S) show near-linear accumulation rates of differing slope, with neither curve reaching saturation over the trial period (72 h). (*B*) Radioactive pulse-chase of flies fed isotope-labeled food for 24 h and then transferred to nonlabeled medium of identical composition. Isotope levels were assayed at three time points: 24 h (immediately before transfer), 48 h, and 72 h. Isotope levels in flies fed diluted food show a faster rate of decline, ruling out that impaired excretion or metabolization of the label underlies the observed differences. Results in *A* and *B* are expressed as an average (± SD) of 3–4 trials, each containing 15 Canton-S males. (*C*) Food consumption measured in the CAFE (4) using YE/S (CM = 10% YE + 10% S; DR = 2.5% YE + 2.5% S) is consistent with radiolabeling results (Fig. 1*B*). The presence of an ad libitum water source in the CAFE chamber did not affect feeding ($P > 0.05$, Student's *t* test). Each time point is expressed as an average (± SD) of four trials, each containing three Canton-S males.

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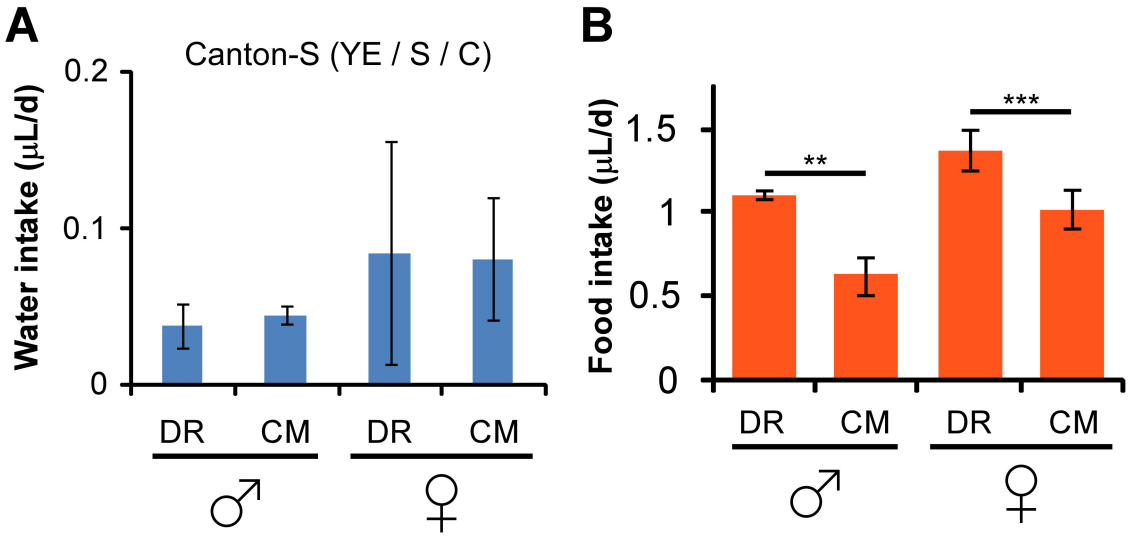

Fig. S2. Food and water intake of Canton-S males on yeast extract/sucrose/cornmeal (YE/S/C) as assayed by isotope radiolabeling (2). (*A*) Water intake per fly over 24 h. Drinking is minimal on both high (CM) and low (DR) yeast concentrations of YE/S/C. (*B*) Food intake per fly over 24 h. Results are expressed as an average (± SD) of 2–6 trials, each containing 6–16 flies. Medium composition is described in Fig. 3. Statistical significance was determined by nonpaired, two-tailed Student's *t* tests between DR and CM media: **, $P < 0.01$; ***, $P < 0.001$.

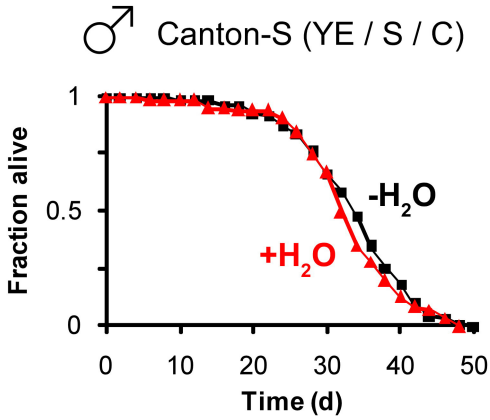

Fig. S3. Water supplementation does not affect the lifespan of Canton-S males on high-yeast (CM) YE/S/C medium. Flies were aged in vials. Medium composition is described in Fig. 3. $n = 87$ flies, -H_2O; 86 flies, +H_2O; $P = 0.40$, log-rank test.

Table S1. Statistics of Cox proportional hazards analysis for the effect of diet on survival in the presence or absence of water

	Effect	df	χ^2	p	Hazard ratio*	Lower CI	Upper CI
YE/S							
Canton-S males	DR	1	10.36	0.0013	1.728	1.244	2.401
	DR+H_2O	1	0.18	0.6694	1.059	0.815	1.376
BY/S							
Canton-S females	DR	1	16.14	0.0001	1.693	1.304	2.199
	DR+H_2O	1	2.55	0.1101	1.284	0.944	1.746
Dahomey females	DR	1	5.32	0.0211	1.450	1.059	1.986
	DR+H_2O	1	0.61	0.4269	0.879	0.640	1.208
YE/S/C							
Canton-S males	DR	1	43.65	<0.0000	3.920	2.569	5.980
	DR+H_2O	1	28.94	<0.0000	2.703	1.871	3.904
Canton-S females	DR	1	23.86	<0.0000	2.478	1.706	3.600
	DR+H_2O	1	30.65	<0.0000	3.188	2.084	4.877

*When the hazard ratio is close to 1, DR has little effect on survival.

Table S2. Dietary restriction sample sizes and lifespan statistics

Flies	Housing	Medium	Conc.	Water access	No. of flies	Mean lifespan, days	Mean DR effect, %	Max lifespan,* days	Max DR effect, %
Canton-S males	Cages	YE/S	CM		68	21.4		29	
(Fig. 2)			DR		81	25.2	18	31	7
			CM	Yes	103	27.4		35	
			DR	Yes	129	27.5	0	35	0
Canton-S males	Cages	BY/S	CM		156	32.1		39	
(Fig. 2)			DR		94	37.0	15	45	15
			CM	Yes	91	39.5		51	
			DR	Yes	79	40.2	2	53	4
Canton-S females	Cages	BY/S	CM		152	29.9		42	
			DR		153	33.6	13	47	12
			CM	Yes	163	38.3		46	
			DR	Yes	153	38.7	1	49	7
Dahomey females	Vials	BY/S	CM		78	53.3		68	
(Fig. 2)			DR		81	59.6	12	74	9
			CM	Yes	78	57.9		72	
			DR	Yes	79	57.6	−1	68	−6
Canton-S males	Cages	YE/S/C	CM		75	42.3		59	
(Fig. 3)			DR		60	56.0	32	73	24
			CM	Yes	77	46.0		63	
			DR	Yes	68	56.9	24	71	13
Canton-S females	Cages	YE/S/C	CM		86	44.7		57	
(Fig. 3)			DR		66	50.1	12	70	23
			CM	Yes	70	42.5		54	
			DR	Yes	61	51.5	21	71	31
Canton-S males	Vials	YE/S/C	CM		86	32.9		42	
(Fig. S3)			DR		87	41.7	27	54	29
			CM	Yes	85	32.0		41	
			DR	Yes	88	42.6	33	53	29

*90th percentile.

Chapter 5

Demographic Mechanisms of *Drosophila* Dietary Restriction

Gil B. Carvalho, William W. Ja, Seymour Benzer and David J. Anderson

Introduction

Dietary restriction (DR), a reduction in nutrient availability short of malnutrition, extends the lifespan of organisms ranging from yeast to mammals (1). It is unclear whether DR acts in different species through similar mechanisms, and thus the relevance of studying DR in model systems remains undetermined.

DR in fruit flies is typically applied by food dilution (2). Since the medium is the only water source, and since *Drosophila* alters its intake according to food concentration (3, 4), medium dilution changes both nutrient availability and hydration. We have shown that the lifespan-extending effect of typical DR paradigms used in flies is entirely abolished in the presence of *ad libitum* water (reference 4, which also constitutes chapter 4 of this thesis). This startling finding suggests that, unlike what has been assumed, classical *Drosophila* DR is mediated by hydration, not nutrient intake. Perhaps more importantly, this demonstrates a fundamental difference between the fly paradigm and mammalian DR, which is not dependent on water availability (5, 6). The most alarming implication of this scenario is that discoveries made in the fly model over the last 13 years may not be relevant for understanding aging and DR in higher organisms.

DR may reduce mortality via two basic mechanisms: an immediate reduction in risk of death or a long-term deceleration in the accumulation of age-related damage (figure 1). A direct test of these theories supported the former model by showing that, in *Drosophila*, acute DR instantly rescues mortality rate to the level of chronic food restriction (7). Previous work in medflies also suggests a potential for full reversion of

mortality upon acute dietary manipulation (8). If this trend is conserved in mammals, it would have an outstanding impact on public health, since instigation of DR at any age would have a full benefit on death risk.

Results and Discussion

We asked whether hydration underlies the reported effect of DR on mortality rate. We have previously shown that CM-fed flies consume water, when available, without changing their food intake (4). As expected, animals had higher age-specific mortality rates when chronically maintained on CM than on DR (figure 2A). We also confirmed that an acute shift to DR resulted in a complete and maintained reversion in mortality to the level of animals on chronic DR (figure 2A), in agreement with published results (7). Strikingly, this reversion was perfectly mimicked when CM-fed flies were given access to *ad libitum* water (figure 2B). Thus, the reported demographic mechanism of DR is entirely dependent on hydration and is likely not conserved in higher organisms, where DR is conducted in the presence of *ad libitum* water (5, 6).

We have previously characterized a DR paradigm that prolongs survival independently from hydration and thus more closely resembles the mammalian phenomenon (4). To elucidate the demographic mechanism of DR in conditions analogous to those used in mammalian studies, we measured the mortality trajectory of animals exposed acutely to the water-independent DR paradigm. Animals switched to DR showed a slower subsequent rise in mortality rate, consistent with slower

damage accumulation, but did not fully revert to the levels of the chronic treatment, in stark contrast to the published findings (7) (figure 3A). To ensure that the conflicting results are not due to genotype differences, we confirmed our observation with the Dahomey wild-type strain (figure 3B). Although Dahomey mortality plateaued late in life on all diets, the overall post-switch trend was identical in both genotypes: deceleration in the increase of death risk without a complete reversion (figure 3).

These results are reminiscent of the effect of temperature, which affects the slope of the mortality trajectory but does not induce a full conversion to the levels of chronic exposure (7), consistent with the notion that thermal history causes accumulation of irreversible damage. Similarly, our results show that, in conditions where water is not a factor, acute DR cannot reverse the damage already sustained by *Drosophila* with a history of exposure to high nutrient levels. These findings are consistent with metabolic theories of aging, which postulate that senescence is a result of the accumulation of toxic by-products of metabolism (9). Finally, our observations predict that acute DR in higher organisms will not confer the full benefits of lifetime exposure, contrary to current belief.

Experimental Procedures

All experiments were conducted as described in reference 4 and chapter 4 of this thesis.

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Figure legends

Figure 1. Mechanistic models for attenuation of mortality rate. Any acute intervention (vertical gray dotted line) may beneficially impact mortality rate in two basic ways. In the short-term model (top), there is a decrease in mortality rate followed by a gradual increase. In the long-term model (bottom), there is a slower rate of increase. The two models are not mutually exclusive. Red line, untreated. Green line, treated chronically. Blue line, treated acutely.

Figure 2. Acute administration of either classical dietary restriction (DR) or *ad libitum* water rescues age-specific mortality rate to the level of flies on a lifelong treatment. **a**, **b**, Canton-S males housed in demography cages and maintained on yeast extract/sucrose (YE/S) concentrated medium (CM) show a permanent shift in their mortality rate to that of flies on the chronic treatment when switched to DR (**a**) or to CM in the presence of a water source (**b**) on day 22. N = 482–658 flies per trial. Dotted vertical lines mark the day when diets were switched.

Figure 3. Age-specific mortality rates in response to midlife-initiation of a water-independent dietary restriction (DR) regime show a reduction in the slope of the mortality trajectory. Canton-S (**a**) or Dahomey (**b**) males housed in vials show a reduction in the slope of the mortality trajectory but not a complete reversion when switched from CM to DR YE/S/C food. Diets were switched when mortality on CM reached ~25% (**c**, day 36; **d**, day 24). **a**, N = 247 flies each for chronic DR and CM; 498 flies for the switch. **b**, N = 478–496 flies per trial. Dotted vertical lines mark the day when diets were switched.

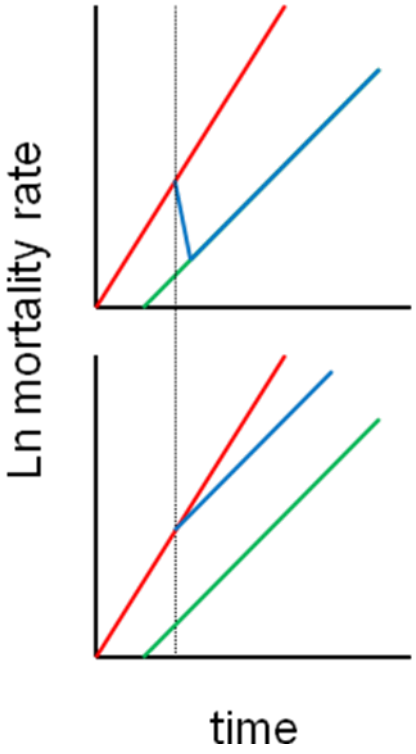

Figure 1

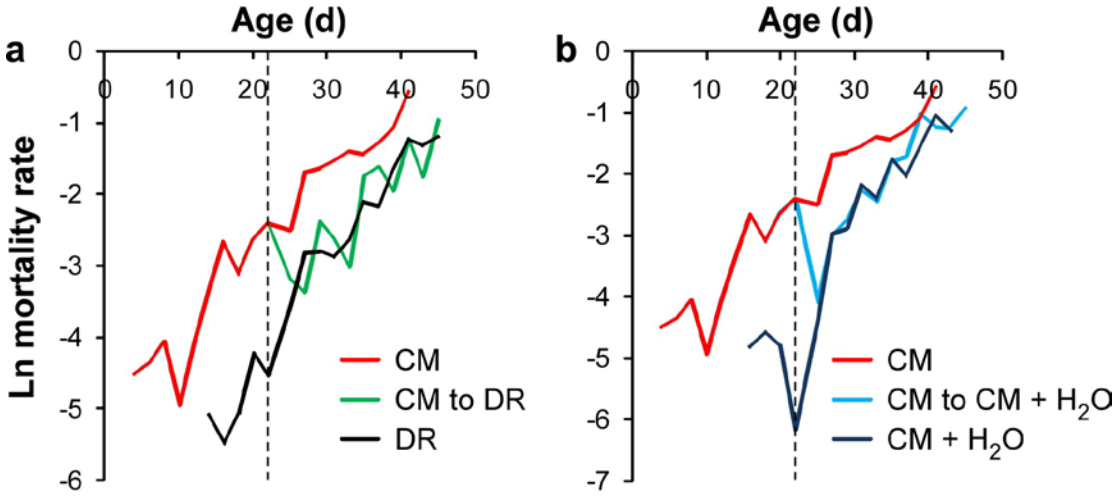
a
Age (d)
Ln mortality rate
0
10
20
30
40
50
-1
-2
-3
-4
-5
-6
CM
CM to DR
DR
b
Age (d)
-7
CM
CM to CM + H2O
CM + H2O

Figure 2

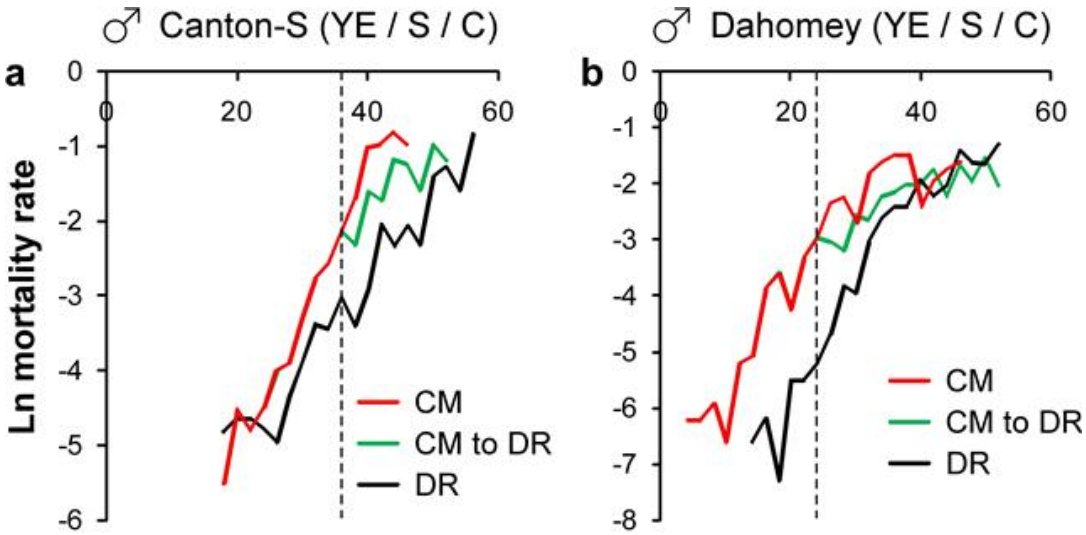
♂ Canton-S (YE / S / C)
a
Ln mortality rate
0
-1
-2
-3
-4
-5
-6
0
20
40
60
CM
CM to DR
DR
♂ Dahomey (YE / S / C)
b
0
-1
-2
-3
-4
-5
-6
-7
-8
0
20
40
60
CM
CM to DR
DR

Figure 3

Chapter 6

The *Drosophila* G Protein-Coupled Receptor, Methuselah, Exhibits a Promiscuous Response to Peptides

This chapter consists of a paper coauthored by William W. Ja, Marisol Madrigal, Richard W. Roberts and Seymour Benzer, which appeared in *Protein Science*. Here, we report two novel activators of the GPCR Methuselah (Mth) in a cell culture system: SP and the non-physiological Serendipitous Peptide Activator of Mth (SPAM).

ACCELERATED COMMUNICATION

The *Drosophila* G protein-coupled receptor, Methuselah, exhibits a promiscuous response to peptides

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Abstract: Methuselah (Mth) is a G protein-coupled receptor (GPCR) associated with longevity in *Drosophila melanogaster*. Previously, Stunted (Sun) was identified as a peptide agonist of Mth. Here, we identify two additional activators of Mth signaling: *Drosophila* Sex Peptide (SP) and a novel peptide (Serendipitous Peptide Activator of Mth, SPAM). Minimal functional sequences and key residues were identified from Sun and SPAM by studying truncation and alanine-scanning mutations. These peptide agonists share little sequence homology and illustrate the promiscuity of Mth for activation. *mth* mutants exhibit no defects in behaviors controlled by SP, casting doubt on the biological significance of Mth activation by any of these agonists, and illustrating the difficulty in applying *in vitro* studies to their relevance *in vivo*. Future studies of Mth ligands will help further our understanding of the functional interaction of agonists and GPCRs.

Keywords: alanine-scanning; GPCR; methuselah; peptide agonists; promiscuity

Introduction

The *methuselah* (*mth*) gene encodes a family B G protein-coupled receptor (GPCR) and is associated with longevity and stress resistance in *Drosophila melanogaster*.[1] Through middle-age adulthood, the *mth*[1] mutant is resistant to loss of the male germline stem cell population[2] and exhibits more robust sensorimotor function,[3] although lifespan extension is sensitive to laboratory conditions.[3–5] Down-regulation of *mth* also affects synaptic transmission in the larval neuromuscular junction, a role that appears distinct from its effects on longevity.[6] We previously isolated antagonists (RWR motif peptides) of Mth that extend lifespan when over-expressed *in vivo*.[7] The interaction site and dynamics of peptide binding to Mth were modeled, describing how these antagonists interact with the Mth ectodomain.[8] Further studies of activators and inhibitors of Mth signaling might lead to a better understanding of GPCR-agonist interactions.

Here, we identify and characterize two new peptide agonists of Mth: *Drosophila* Sex Peptide (SP) and Serendipitous Peptide Activator of Mth (SPAM).

†Deceased

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Agonists of Mth, including the previously identified Stunted (Sun) peptide,[9] share minimal sequence homology, suggesting a remarkable promiscuity of Mth for activation. As *mth* mutants show no defects in SP-controlled behaviors, the physiological relevance of these interactions remains unclear. These peptides should provide new tools for probing the activation of class B GPCRs, a family associated with several human diseases.[10]

Results and Discussion

Characterization of a novel peptide agonist of Mth

Previously, a peptide derived from the N-terminus of Stunted (N-Sun) was identified as an agonist for Mth by screening fractionated *Drosophila* homogenates on HEK 293 cells stably expressing Mth and measuring intracellular calcium mobilization.[9] We subsequently used mRNA display selection to identify novel RWR motif-containing peptides that bind with high affinity to Mth and act as inhibitors of Sun-mediated Mth activation.[7] For two of the peptide antagonists, R8-01 and R8-12, we synthesized randomly scrambled mutants for use as negative controls. As expected, the scrambled R8-12 peptide exhibited no activity on Mth. Surprisingly, however, robust calcium mobilization was observed upon addition of the scrambled R8-01 peptide to Mth-expressing cells [Fig. 1(A)], whereas no activity was seen in nontransfected control cells. We subsequently named the R8-01 scrambled peptide, "Serendipitous Peptide Activator of Mth" (SPAM). SPAM has no homology with N-Sun and appears to be a more potent Mth agonist [EC_{50} of 2.5 μM compared with 11 μM for N-Sun, Fig. 1(B)]. SPAM was inhibited by Mth peptide antagonists, providing further evidence that activation is specific for Mth [Fig. 1(C)].

To identify motifs necessary for agonist activity, a series of 15-mer peptides for N-Sun and 12-mers for SPAM were synthesized and tested in the cell-based calcium mobilization assay. For N-Sun, the region of agonist activity was localized to the N-terminus, and the minimal active peptide sequence identified was AWRAAGITYIQYS [Fig. 2(A)]. For SPAM, the minimal functional peptide with high activity was LQAPRRSVMRW [Fig. 2(A)]. Since shorter peptides in the N-terminal region of N-Sun were not tested, it is possible that significantly shorter peptides could be derived from N-Sun that retain full activity.

To identify the residues critical for Mth agonist activity, a series of alanine-scanning mutants for the minimal functional sequences of N-Sun and SPAM were assayed. These experiments revealed little overlap between the key residues in N-Sun and SPAM, except for the single Trp in each peptide, which was important for signaling [Fig. 2(B)]. Although N-Sun and SPAM differ greatly in their primary sequences, they may present the Trp residue similarly to the receptor during Mth activation. Further studies of the mutant peptides should determine the quantitative contributions of individual amino acids on agonist affinity and efficacy.

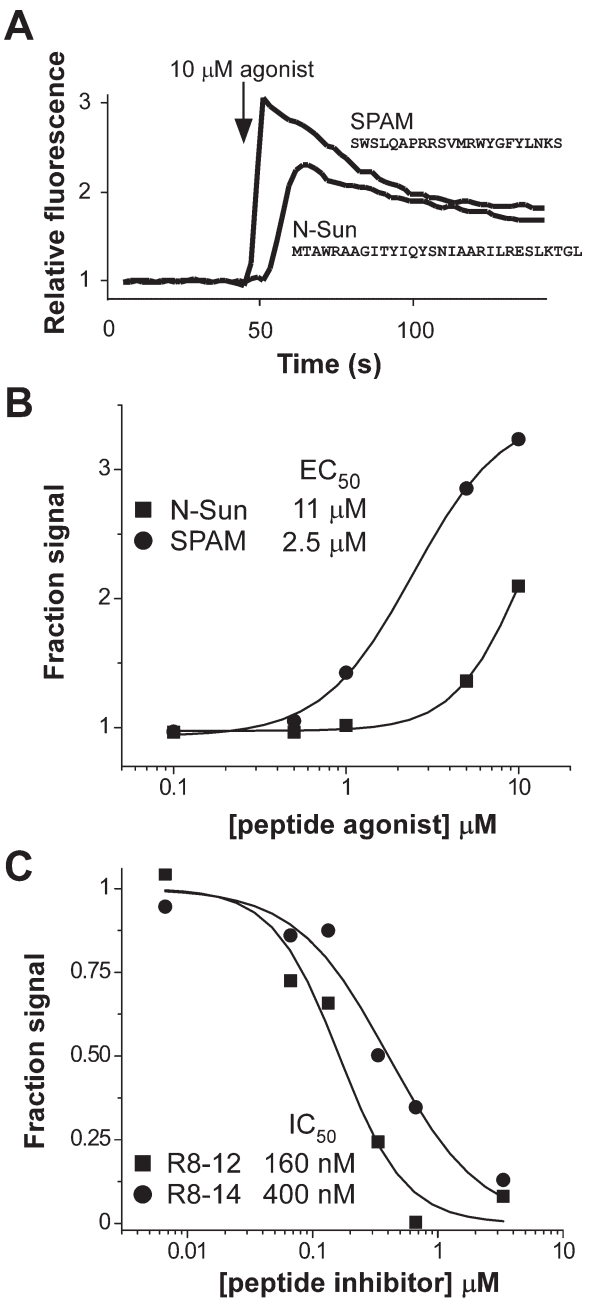

Figure 1. SPAM is a Mth agonist. (A) Application of 10 μM N-Sun or a scrambled variant of R8-01 (SPAM) at the indicated time results in mobilization of intracellular calcium and increased fluorescence in HEK-Mth cells. (B) Concentration dependence of Mth activation by N-Sun (■) and SPAM (●) shows SPAM is a more potent agonist. (C) RWR motif peptides R8-12 (■) and R8-14 (●), previously shown to inhibit N-Sun activation of Mth, also antagonize SPAM-mediated Mth signaling. The maximum fluorescence values after the addition of SPAM agonist (10 μM final) to HEK-Mth cells preincubated with varying concentrations of R8-12 or R8-14 are expressed as a fraction of the fluorescence observed in the absence of antagonists.

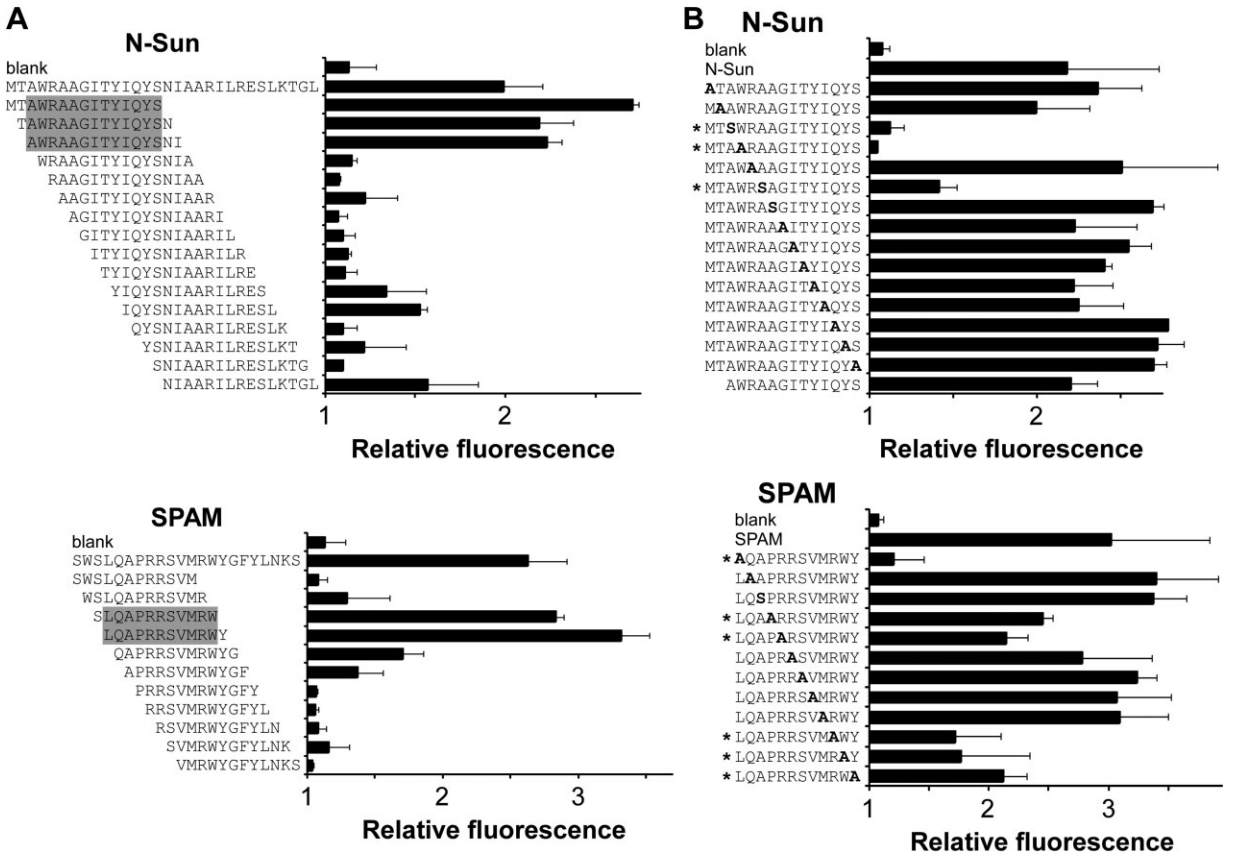

Figure 2. Identification of minimal sequences and critical residues for Mth activation. (A) A series of 15-mer peptides derived from N-Sun and 12-mer peptides derived from SPAM were tested for their ability to activate Mth in the cell-based calcium mobilization assay. Reported values (± s.d.) represent the maximum fluorescence achieved after the addition of peptide divided by the baseline average. The shaded region highlights the putative minimal peptide agonist. "Blank" is a negative control where buffer without peptide was added. (B) A series of alanine-scanning mutants were tested as in (A). Each residue in the minimal functional N-Sun and SPAM peptides was mutated to Ala and assayed. Wild-type Ala residues were mutated to Ser. All peptide sequences listed have an additional C-terminal glycine (not shown), and were tested at a concentration of 10 μ*M* (N-Sun peptides) or 5 μ*M* (SPAM peptides).

Sun is the ε subunit for the eukaryotic mitochondrial ATP synthase.[11] Curiously, the functional 15-mer sequence of N-Sun for Mth activation corresponds well to the most conserved region of the peptide, in comparison with the ε subunit genes of other organisms including plants and mammals.[12] The *mth* family of GPCRs, however, has thus far been found only in insects.[13,14] Further studies will help to elucidate whether Sun functions both in and out of the mitochondria and verify its physiological interaction with Mth.

Identification of SP as a Mth agonist

The activation of Mth by SPAM, a non-physiological peptide with a randomly generated sequence, suggested that Mth has remarkably low ligand specificity. To further test this hypothesis, we challenged Mth-expressing HEK cells with other unrelated peptides and found that SP also elicited activation [Fig. 3(A)]. Agonist activity was eliminated when the SP sequence was scrambled or when the four C-terminal residues critical for SP-mediated behavioral effects[15] were removed. SP did not induce calcium signaling in HEK 293 cells expressing the *mth-like* (*mthl*) receptors, *mthl1, mthl2, mthl3*, and *mthl5*, suggesting high specificity for Mth, although whether the *mthl* receptors are coupled to calcium mobilization in these cell lines is unknown.

To test the physiological relevance of the SP-Mth interaction, we generated a *mth* null mutant using FLP-FRT recombination,[16] resulting in a line with a defined chromosomal deletion, mth^{Δ}, encompassing all of the protein coding exons of *mth*. Homozygous mth^{Δ} flies are embryonic lethal, which is consistent with published data suggesting that *mth* is an essential gene.[1] Virgin female flies heterozygous for mth^{Δ} showed a approximately two-fold reduction in mRNA transcript levels of both *mth* splice variants [Fig. 3(B)], supporting the view that mth^{Δ} is a null allele. Previous studies have relied on a hypomorphic P-element insertion allele in the 3rd intron of *mth*. This homozygous insertion mutant (mth^{1}) showed ~10-fold and ~25-fold reductions in *mth-A* and *mth-B*,

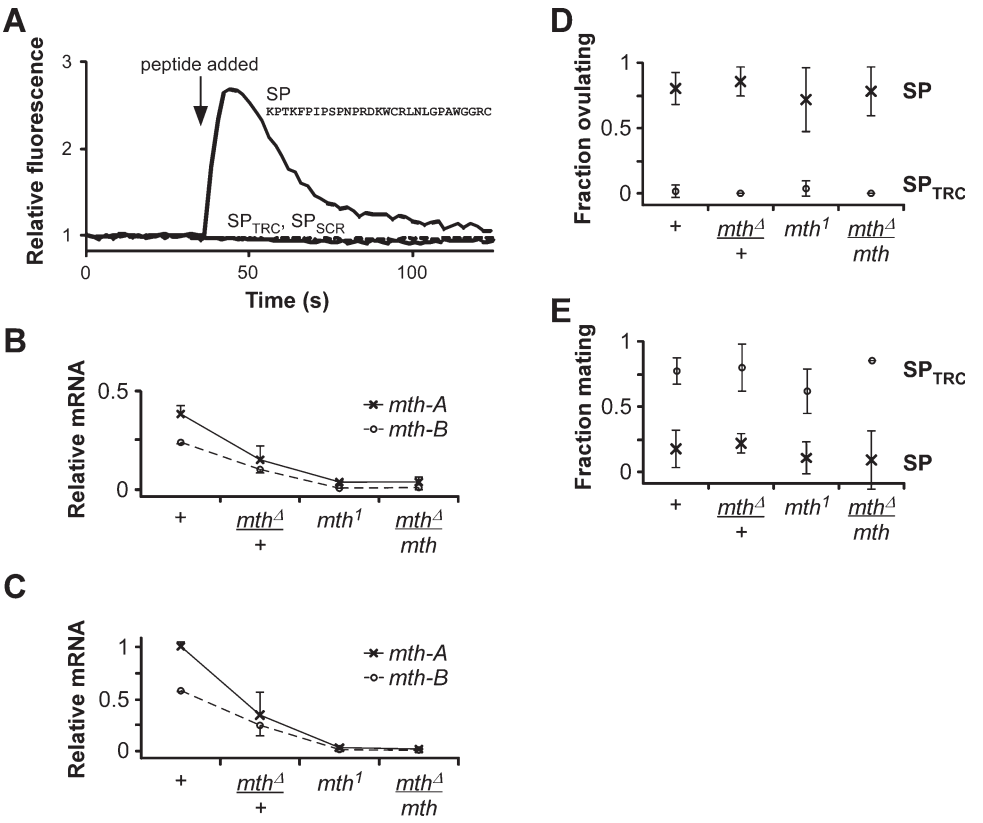

Figure 3. *Drosophila* Sex Peptide (SP) activates Mth. (A) Application of 10 μ*M* SP induces mobilization of intracellular calcium and increased fluorescence in HEK 293 cells transiently transfected with *mth-B*. SP_{TRC} (solid grey line), which lacks the C-terminal four residues of SP, and SP_{SCR} (PWPKC NNGIP ARRGF KSLRT PPGCL PWDK, dashed line), a scrambled version of SP, do not activate Mth. (B, C) Relative transcript levels of the *mth-A* (×) and *mth-B* (○) splice variants in different *mth* mutant alleles from RNA isolated from (B) virgin female adults (1–2 day old) or (C) wandering 3rd instar larvae. Results are averages (± s.d.) from two independent collections of total RNA (biological replicates). (D, E) *mth* mutants exhibit no defects in SP-induced behaviors. (D) Injection of SP (×) into virgin females dramatically increases ovulation in the control and mutants of *mth*. (E) Injection of SP (×) into virgin females greatly reduces sexual receptivity to males. Injection of the negative control peptide, SP_{TRC} (○), had a negligible effect on ovulation and mating. Behavioral results are averages (± s.d.) from two to seven independent experiments, each consisting of 6–25 flies.

respectively. Animals bearing mth^{Δ} *in trans* to mth^{1} were viable and showed no obvious morphological or behavioral defects. Transcript levels in these flies were similar to levels in the homozygous mth^{1} mutant. qPCR analysis of total RNA from 3rd instar larvae showed similar ratios of *mth* transcript levels in all alleles [Fig. 3(C)], although *mth* mRNA levels were higher in larvae, consistent with previous microarray results.[17]

In *Drosophila*, mating strongly affects female behavior. This post-mating response, which includes stimulated ovulation and reduced sexual receptivity, is controlled by SP, a protein produced by the male accessory glands and transferred in the seminal fluid during copulation.[15] We thus assayed behaviors associated with the postmating response in the *mth* alleles. Virgin female mutants exhibited normal ovulation and mating frequencies after SP injection [Figs. 3(D,E)]. The Sex Peptide Receptor (CG16752) is known to mediate the role of SP in triggering the postmating response.[18] Hence, SP either does not interact with Mth *in vivo*, or the downstream effects are subtle and/or uncharacterized.

Conclusion

We have characterized two peptide agonists for Mth—SPAM and SP—that are of similar or higher potency than the previously identified Sun agonist, show specificity for Mth over Mth-like receptors, and are inhibited by the RWR motif antagonist peptides. The promiscuity of Mth casts doubt on the relevance of the agonists *in vivo*, and cautions against the broad interpretation of *in vitro* GPCR studies. However, despite the lack of support for physiological interactions of the agonists with Mth, recent studies of other promiscuous GPCRs with potential roles in nutrient-sensing and immune response[20,21] hint at a possible function of Mth as a peptide sensor *in vivo*. The identification of a *bona fide* signaling partner for Mth, as well as the discovery of agonists for the related Mth-like receptors, will help elucidate the role of this GPCR family in *Drosophila* aging, health, and development.

Materials and Methods

Peptide synthesis

N-Sun (MTAWR AAGIT YIQYS NIAAR ILRES LKTGL) and SPAM (SWSLQ APRRS VMRWY GFYLN KS) were synthesized in-house on a 432A Synergy peptide synthesizer (Applied Biosystems, Foster City, CA) using standard Fmoc chemistry. *Drosophila* SP (KPTKF PIPSP NPRDK WCRLN LGPAW GGRC), a control peptide lacking the C-terminal four residues (SP_{TRC}), and a scrambled version of SP (PWPKC NNGIP ARRGF KSLRT PPGCL PWDK, SP_{SCR}) were synthesized by Bio-Synthesis, Inc. (Lewisville, TX) and provided on-resin. Synthetic peptides were cleaved from the resin and deprotected by agitation in trifluoroacetic acid (TFA):1,2-ethanediol:thioanisole (90:5:5) for 2 h at room temperature. Crude peptides were desalted by precipitation in methyltertbutyl ether. Peptides were purified by reversed-phase HPLC (C18, 250 × 10 mm, Grace Vydac, Hesperia, CA) on an aqueous acetonitrile/0.1% (v/v) TFA gradient. Peptide masses and the formation of the intramolecular disulfide bridge in SP were confirmed by MALDI-TOF mass spectrometry. Antagonist peptides, R8-01 (MNVSW GSFPS SWLQR YYLAK RR), R8-12 (MRLVW IVRSR HFGPR LRMA), and R8-14 (MAPRA VWIQR AIQAM FRLA), were obtained as described previously.[7] All peptides were soluble in ddH_2O or 1× PBS, and concentrations were determined by measuring absorbance at 280 nm.

Peptide truncation and alanine-scanning mutagenesis series were synthesized with C-terminal glycine residues by JPT Peptide Technologies GmbH (Micro-Scale Peptide Sets, Berlin, Germany). Crude, dried peptides were provided in 96-well plates at ~50 nmol of full-length peptide per well. Peptides were reconstituted in 20 μL of dimethyl sulfoxide prior to their use in cell signaling assays.

Cell-based calcium signaling assay for Mth activation

Mth activation was measured by observing calcium mobilization essentially as described using HEK 293 cells stably expressing the B splice variant of *mth* (HEK-Mth cells) and the fluorescent calcium indicator, Fluo-4 AM (Molecular Probes, Eugene, OR).[7] Data analysis and background subtraction were performed with Softmax Pro 4.8 (Molecular Devices, Sunnyvale, CA) and sigmoidal fits were calculated using Origin 6.0 Professional (OriginLab Corp., Northampton, MA). Assays of *mth-B* and *mth-like* (*mthl*) receptors were also performed using either transiently transfected (Lipofectamine, Invitrogen, Carlsbad, CA) or isogenic stable expression (Flp-In system, Invitrogen) HEK 293 cell lines generated with cDNA clones from the *Drosophila* Genomics Resource Center (*mth-B*, SD05804; *mthl1*, AT18671; *mthl2*, RH57551; *mthl3*, GM02553; and *mthl5*, RE31350). cDNA was cloned into the pcDNA3.1+ or the pcDNA5/FRT vectors (Invitrogen) for transient or Flp-In transfections, respectively, using sticky-end PCR[19] with parental plasmid-specific primers.

Generation of a mth *null line*

To generate a defined chromosomal deletion between the P-element insertion lines flanking the *mth* gene, *e03119* and *d05374*, we used FLP-FRT recombination, as described previously.[16] Briefly, flies bearing heat-shock driven FLP recombinase and both the *d05374* and *e03119* P-element insertions *in trans* were heat-shocked to induce expression of FLP recombinase, resulting in the generation of chromosomal deletions that were identified by loss of a marker (eye color). Deletion lines generated in this way bear a new hybrid transposon combination containing fragments from both *e03119* and *d05374*. Hence, the resulting lines were confirmed by genomic PCR with transposon-specific primers (RB3′-in: 5′-TGC ATT TGC CTT TCG CCT TAT and XP5′-in: 5′-AAT GAT TCG CAG TGG AAG GCT) that generate a fragment of known size across the newly formed hybrid element. The homozygous-lethal deletion line (mth^{Δ}) was established and maintained over the *TM6B* balancer. Assays with mth^{Δ} were performed by comparing heterozygotes (mth^{Δ}/w^{1118}) with transheterozygotes (mth^{Δ}/mth^{1}), where the mth^{1} mutant was extensively backcrossed (>10 generations) with the control line, w^{1118}.

Quantitative real-time PCR

Total RNA from wandering 3rd instar larvae or virgin female adults (1–2 day old) was purified using TRIzol (Invitrogen), following the manufacturer's instructions. Approximately 10–20 animals were homogenized per replicate. Samples were treated with DNase I (Invitrogen), before being used for reverse transcription.

First-strand cDNA was synthesized with random nonamer primers in a final reaction volume of 20 μL. Total RNA (2 μg), primers (N_9, 60 pmol), and dNTP (20 nmol each) were incubated at 65°C for 5 min followed by a quick chill on ice. Reverse-transcription buffer (1× final) and SUPERase-In (1 U/μL final, Applied Biosystems/Ambion, Austin, TX) were added and the reaction was incubated at room temperature for 2 min. Reverse transcriptase (Superscript II, 200 U, Invitrogen) was added and the reaction was incubated at 25°C for 10 min to initiate cDNA synthesis. Incubation temperature was ramped up and maintained at 42°C for 1 h prior to inactivating the reaction by heating at 70°C for 15 min. Negative control reactions were treated identically but did not contain reverse transcriptase.

Quantitative real-time PCR was performed on an iCycler iQ5 system (Bio-Rad, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad) with 1 μL of cDNA

(100 ng RNA equivalent) and a final primer concentration of 0.25 μ*M* in a total volume of 20 μL. Splice variants of *mth* were differentiated using exon-specific reverse primers (mthA-RP: 5′-CAC TGT TGT TTA CCT CCT CAC CCT and mthB-RP: 5′-TTC CCA CGG TAA TAC GAC TTG CCA) with a common forward primer (mthAB-FP: 5′-ACC AAA CTT GGG CCA ACG TCT TTC). Cycling conditions were 3 min at 95°C followed by 40 cycles of 95°C for 10 s (denaturation) and 60°C for 45 s (annealing and extension). Melting curves were generated after each qPCR run, and final PCR products appeared to be the correct size when analyzed by agarose gel electrophoresis. qPCR of *Actin 88F* (primers 5′-GAT CAC CAT TGG CAA CGA and 5′-TCT TGA TCT TGA TGG TCG) was performed as a positive control on all samples. To compare transcript levels across different life stages and/or age groups, qPCR results were normalized to input total RNA.

Behavioral assays

Peptides (~200 nL of 240 μ*M* in ddH_2O) were injected into the thorax of CO_2-anesthetized, 4- to 6-day-old virgin females. Flies recovered in food vials for ~8 h and were subsequently assayed for the presence of an egg in the uterus by gently squeezing the tip of the abdomen with forceps. After 8–12 h, individual females were transferred without anesthesia to circular mating chambers (1 cm diameter, ~0.6 cm height) containing 1 virgin, control (Canton-S) male and observed for 1–2 h. Courtship was confirmed for males in each chamber, and successful copulation was scored. Data from multiple independent experiments were averaged and shown ± s.d.

Acknowledgments

The authors thank the Bloomington *Drosophila* Stock Center for FRT-related fly lines (#7, 7758, and 8136), the Exelixis Collection at Harvard Medical School for P-element insertion lines (*e03119* and *d05374*), the *Drosophila* Genomics Resource Center for cDNA clones, and X.-Y. Huang (Cornell University Weill Medical College) for the HEK-Mth cell line.

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Chapter 7

Nonlethal PCR Genotyping of Single *Drosophila*

This chapter consists of a paper coauthored by William W. Ja and Seymour Benzer, which appeared in *Biotechniques*. We describe a method for DNA isolation and amplification from small biological samples, such as the legs or wings of a single fly. Since it does not require the animal to be sacrificed, it allows genotyping of candidate recombinants originated from P-element mobilization or chromosomal recombination one generation earlier than with classical methodology and, importantly, before stocks are established.

Non-lethal PCR genotyping of single Drosophila

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Supplementary protocol is available online at www.BioTechniques.com.

In Drosophila, genetic techniques relying on stochastic chromosomal rearrangements involve the generation and screening of a large number of fly stocks to isolate a few lines of interest. Here, we describe a PCR-based method allowing non-lethal molecular characterization of single flies. Using this procedure, individual candidate recombinant animals can be genotyped and selected one generation earlier than with extant methodology and, importantly, before stocks are established. This advance should significantly facilitate several of the most fundamental and routine techniques in Drosophila genetics.

Chromosomal recombination (CR) and mobilization of transposable elements (MTE) are two fundamental techniques in Drosophila genetics. CR is commonly used to associate or dissociate two alleles on different loci of a chromosome. MTE generates two classes of excisions: (*i*) precise (also called revertants), where the transposon is completely removed leaving a wild-type chromosome; and (*ii*) imprecise, where rearrangements occur to the transposon and/or the surrounding sequence (1). These methods have two major limitations. First, they rely on largely unpredictable chromosomal rearrangements. Hence, reliable characterization of the resulting DNA structure requires molecular genotyping, usually by polymerase chain reaction (PCR) and DNA sequencing. Second, these methods are generally inefficient. Since recombination frequency is proportional to the distance between the two loci of interest, achieving CR between two close loci requires screening a large number of candidate animals. Similarly, for MTE, isolating an imprecise excision causing a deletion in a given gene may require screening hundreds of lines, particularly when dealing with a large locus. These genetic experiments therefore typically involve generating single animals that bear unique chromosomal rearrangements, from which tens or even hundreds of individual stocks are derived and screened (1). Excisions isolated through a visible marker (such as eye color) and candidate recombinants can sometimes be screened phenotypically. However, phenotypes are often too burdensome for screening or unknown altogether (such as in the case of reverse genetics). In these cases, screening relies on molecular techniques. A procedure allowing non-lethal genotyping of single animals would allow direct molecular screening of first-generation candidate recombinants (i.e., at least one generation earlier than with classic methodology), with stocks established from only a few interesting individuals. Such a method should contribute to making CR and MTE efforts substantially faster, cheaper, and less burdensome. Here, we describe a PCR-based protocol for genotyping single Drosophila fruit flies using small body parts. Our technique allows DNA purification and amplification from the wings of a single, live fly with no significant impairment of robustness or reproductive ability.

DNA can be purified from a whole, single fly (2). We found that this protocol is also effective when using a single head or thorax, but not smaller body parts (Figure 1A and data not shown) and therefore requires the genotyped animal to be sacrificed. We developed a protocol allowing DNA purification from a pair of fly legs. The two forelegs of an anesthetized male were sectioned at the proximal femur, placed in a 0.2-mL PCR tube (Sorenson Bioscience, Salt Lake City, UT, USA) and covered with 10 µL of 400 µg/mL protease K (MP Biomedicals, Solon, OH, USA) in buffer A (10 mM Tris-Cl at pH 8.2, 1 mM EDTA, 25 mM NaCl). Optimal results were obtained when the biological sample was entirely submerged in the buffer. Importantly, homogenization was not necessary in these conditions and sufficient DNA was obtained by virtue of the protease digestion. The tubes were incubated at 37°C for 1 h and then at 95°C for 2 min to inactivate the protease. A 540-bp fragment of the Drosophila *actin* gene (*Act42A*) was amplified using the following PCR conditions: 1× iProof HF buffer (Bio-Rad, Hercules, CA, USA), 0.2 mM dNTPs, 0.5 µM primers (forward: 5′-GGTCGCGATTTAACCGACTACCTGAT-3′; reverse: 5′-CTCTTGCTTCGAGATCCACATCTGCT-3′), 3 µL fly leg DNA template and 0.4 U iProof High-fidelity DNA polymerase (Bio-Rad) in a total volume of 20 µL. Thermocycler conditions were 1 cycle of initial denaturation (98°C for 30 s); 35 cycles of denaturation (98°C for 10 s), annealing (63°C for 15 s), and extension (72°C for 20 s); and 1 cycle of final extension (72°C for 10 min). Running 5 µL of each reaction on an agarose gel revealed a single band of the expected size and similar in intensity to the product obtained from head DNA (Figure 1A, lanes 5 and 6).

Although this method isolated DNA suitable for PCR from a small body part, leg amputation is an invasive procedure that likely affects fitness. Since the goal of non-lethal genotyping is to select animals

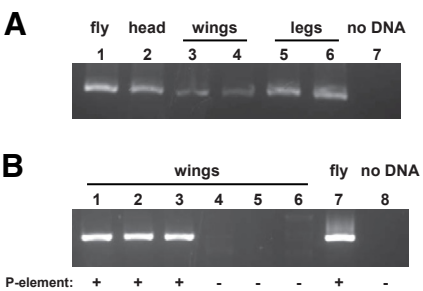

Figure 1. PCR amplification of DNA isolated from discrete body parts of a single fly. (A) DNA extracted by either the lethal purification method (see Reference 2) (lanes 1–2) or our non-lethal protocol (lanes 3–6) was PCR-amplified using primers flanking a 540-bp fragment of the *Act42A* gene. Biological samples: lane 1, whole fly; lane 2, single head; lanes 3–4, pair of wings; lanes 5–6, pair of legs; lane 7, no template. (B) DNA purified from wings of flies bearing the UAS-dSOD2 P-element insertion (lanes 1–3), but not control flies (lanes 4–6), allows PCR amplification of a product specific to the P-element. Lane 7, PCR from DNA extracted from a whole fly bearing the P-element. Lane 8, control reaction with no template added.

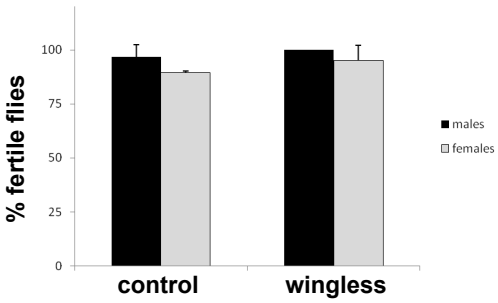

Figure 2. Wing ablation does not affect the fertility of males or females. Wingless or control flies were housed with one animal of the opposite sex in culture vials for 7 days and scored for viable progeny. *n* = 20–30 animals per group. Error bars, ±SD.

with which to establish a stock, flies must not only survive genotyping but also remain robust and fertile. We therefore proposed that fly wings could be used as the biological sample. Our online protocol successfully isolated enough DNA via a double wing ablation (a pair of fly wings, sectioned immediately distal to the wing base) to yield a visible band after PCR (Figure 1A, lanes 3 and 4). Increasing the concentration of protease was not beneficial, and shortening the incubation time reduced extraction efficiency (data not shown). As seen previously with leg-derived DNA, the results were most consistent when the wings were fully covered by the protease solution. Overall, using two wings for the extraction gave optimal reproducibility.

To test the specificity of the amplification from wing DNA, we PCR-amplified a sequence specific to the UAS-dSOD2 transgenic insertion (3) (the primers used were forward: 5′-AGTACTGTCCTCCGAGCGGA-3′ and reverse: 5′-TAGGGCAGCTTCGGTAGGGT-3′). A PCR product was obtained from DNA extracted from wings of UAS-dSOD2 flies, but not from controls lacking the transgene (Figure 1B).

We next investigated if double wing ablation affected reproductive ability. The courtship ritual of Drosophila males includes unilateral wing vibrations thought to influence female receptivity (4). We tested the ability of wingless flies to generate viable progeny when housed with a winged cohort of the opposite sex. The performance of wingless animals was indistinguishable from winged controls (Figure 2). This result demonstrates that double wing ablation does not affect long-term reproductive ability and constitutes a convenient non-lethal genotyping method.

A method for non-lethal genotyping has been described in the honeybee (5). The advent of a protocol designed specifically for non-lethal genotyping in Drosophila should significantly facilitate molecular genetics in this well-established genetic model organism. To a large extent, non-lethal PCR can replace current molecular methods in the context of fly genetics with a significant saving in time and cost, since it allows genotyping at least one generation earlier [i.e., before stocks are established (Figure 3)]. Candidate animals can be screened molecularly one by one or in small batches until a chromosomal event of interest is isolated, virtually eliminating unnecessary labor and reducing reagent costs. As with current methodology, the number of animals screened before interesting stocks are isolated varies from experiment to experiment. In practice, since a recombinant between two alleles located on different chromosomal

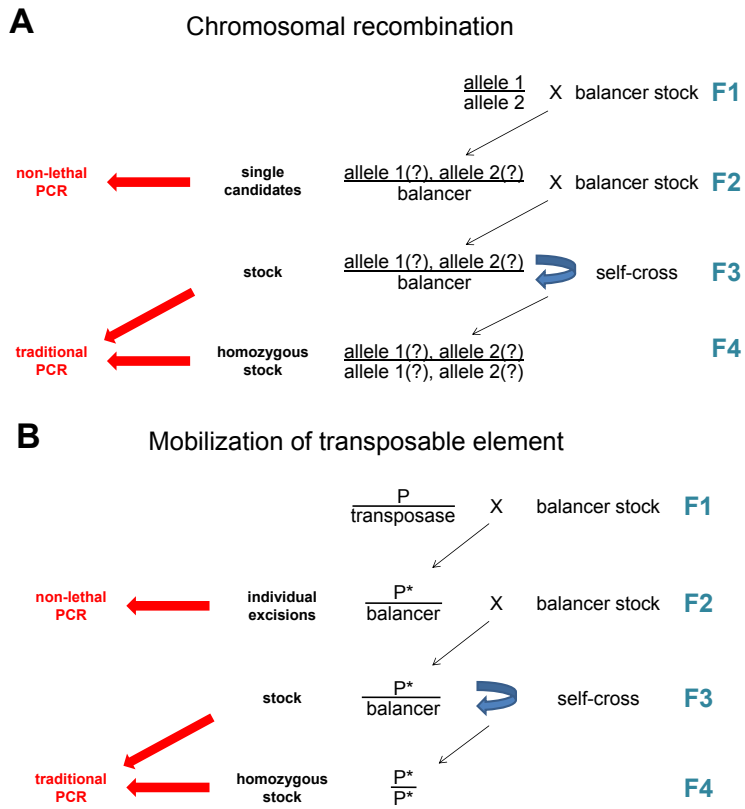

Figure 3. Non-lethal genotyping allows molecular characterization before stocks are established. (A) Chromosomal recombination. F1: individuals bearing the two alleles to be recombined in trans are crossed to a balancer stock. F2: candidate recombinants are isolated as individual female progeny and balanced to generate stocks. Non-lethal PCR allows genotyping at this stage. F3: balanced stocks of candidate recombinants are self-crossed to generate homozygous stocks. Traditional PCR allows genotyping at this stage or F4. F4: homozygous stocks. (B) Mobilization of transposable element. F1: flies bearing transposable element (P) in trans to a source of transposase are crossed to a balancer stock. F2: numerous individual animals bearing different excision events (P*) are isolated (typically on the basis of defective eye color) and balanced to generate stocks. Non-lethal PCR allows genotyping at this stage. F3: balanced excision stocks are homozygosed. Traditional PCR allows genotyping at this stage or F4. F4: homozygous excision stocks.

arms occurs, on average, 50% of the time, it often suffices to genotype two or three candidates to isolate such a line, with this number increasing as the distance between the alleles diminishes. As for MTE, the frequency of a particular excision event is a function of the properties of each transposon and its insertion site and ranges from very frequent (e.g., precise excisions of Piggybac elements, typically requiring only one or two candidates to be genotyped) to very infrequent (e.g., an imprecise excision resulting in a null allele of a large locus, which may require several hundred candidates to be screened).

Since first-generation recombinants bear the putative rearrangement—a recombination in CR and an excision in MTE—over a balancer chromosome, the PCR product should be unique to the chromosome of interest. Specifically, our method is useful in CR experiments to confirm the recombination of insertions of known sequence (by either using two primers complementary to the insertion or one on the insertion and another on the flanking genomic region) and deletions (using flanking primers). For MTE, useful primer sets include those with a primer on one end of the transposon and another on the neighboring genomic region, as well as primers flanking the insert, which can identify imprecise excisions where the size of the amplicon is reduced upon deletion of part of the endogenous chromosome.

Our protocol is particularly useful for identifying local transpositions, a specific case of MTE that takes advantage of the propensity of transposable elements to hop locally. The goal of local transposition is to mobilize an insert into a specific nearby gene of interest (6). Candidate animals can be effectively screened using a primer on one end of the insertion coupled with a set of primers in the target gene.

Note added in proof: It has been brought to the attention of the authors that a similar method has been previously described in a non–peer-reviewed, non-indexed publication by Gleason et al. [Gleason, J.M., K.A. Cropp, and R.S. Dewoody. 2004. DNA preparations from fly wings for molecular marker assisted crosses. Drosophila Info. Serv. *87*:107-108.]

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Supporting Information

DNA isolation and amplification from Drosophila wings

PROTOCOL FOR:
Non-lethal PCR genotyping of single Drosophila

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LEGEND

* ***HINT***

REST

REAGENTS

- Buffer A (10 mM Tris-Cl at pH 8.2, 1 mM EDTA and 25 mM NaCl)
- Protease K (MP Biomedicals, Solon, OH, USA) (400 μg/mL in Buffer A, prepared fresh from a 20 mg/mL frozen stock)
- iProof high-fidelity DNA polymerase and iProof HF buffer (Bio-Rad, Hercules, CA, USA)
- dNTPs
- Custom primers

PROCEDURE

1. Anesthetize flies to be genotyped and section both wings immediately distal to the base (exact location is not critical but using most of the wing will ensure optimal results) with a razor blade.
2. Place wing pair at the bottom of a 0.2-mL PCR tube and carefully cover with 10 μL protease K (400 μg/mL in buffer A).

* *Ensure that wings remain submerged in the solution. Due to their hydrophobicity, wings will float if added to the solution already in the tube. Transferring the wings to the dry tube and covering with the solution circumvents this problem.*

3. Incubate at 37°C for 1 h.
4. Inactivate the protease K by incubating at 95°C for 2 min.

5. PCR-amplify gene of interest. Thermocycler conditions: 1 cycle of 98°C for 30 s; 35 cycles of 98°C for 10 s, Tm+3°C for 15 s, 72°C for 15s/kb; 1 cycle of 72°C for 10 min.

Reagent	**final concentration**
iProof HF buffer (Bio-rad)	1×
dNTPs	0.2 mM each
custom primers	0.5 μM
DNA template	3 μL of wing extract solution
iProof high-fidelity DNA polymerase	0.4 U
ddH2O	to a total volume of 20 μL

6. Run 5 μL of each reaction on an agarose gel.

EQUIPMENT

- Single-edged no. 9 industrial razor blades (Surgical Carbon Steel) (Cat. no. 55411-050; VWR, West Chester, PA, USA)
- 0.2-mL Ultra tubes for PCR (Cat. no. 1695; Sorenson Bioscience Inc. Salt Lake City, UT, USA)
- PTC-200 Peltier Thermo Cycler (MJ Research, St. Bruno, Quebec, Canada)

Conclusion

1. Measuring feeding in *Drosophila*

The methodology we developed allows an unprecedented insight into fly feeding behavior. The two techniques are highly complementary. Radiolabeling is extremely sensitive and can be used to assay ingestion in the exact conditions used to raise and age flies. On the other hand, it requires sacrificing the animals and thus allows only one measurement per experimental group. This has drawn some criticism, since organismal capacity for the label may confound the interpretation if recordings are made past the linear accumulation phase (1). This issue can be addressed through time-course trials (2-4). We have shown that isotope accumulation is near linear for at least 72 h, validating our 24 h recordings (2). Another caveat of radiolabeling is that isotope levels are a product of ingestion, absorption, excretion and metabolization of the label. The latter two parameters can be ruled out through the use of pulse-chase trials (3, 4), but ingestion and absorption cannot be unambiguously deconvoluted (5). In conclusion, radiolabeling allows sensitive measurements, but it does not distinguish between the behavioral (i.e., ingestion) and the metabolic (i.e., gut absorption) components of intake.

The CAFE was developed largely to circumvent these issues. As a direct, unambiguous measurement of ingestion, it is instrumental in addressing the behavioral component of feeding. Measurements can be made continuously, in real time, since animals need not be killed. Feeding can thus be monitored, in the same population, before and after a dietary or environmental manipulation is introduced. As an added benefit, the CAFE assays undisturbed animals (6). The main shortcoming of this

method is that the conditions—liquid diet, no egg-laying surface—differ from the normal culture environment.

In our experience, the two assays tend to show similar results. Compensatory feeding, higher feeding rate in females and the effect of mating on female intake can all be seen with both radiolabeling and the CAFE (2–4 and our unpublished data). Employing both methods thus constitutes the most reliable way to characterize a feeding phenotype.

Historically, the most widely used feeding assay in fruit flies has been dye labeling. The main problem with this technique is that the non-absorbable dyes commonly used start being excreted after a short period (30–60 mins), making dye labeling a useful assay of short-term ingestion, but uninformative in the long term. Unfortunately, this fact is often overlooked and researchers continue to generate, publish, and often base entire studies on misleading recordings (1, 7–9). Although our methodology has begun to be embraced by the fly community (10, 11), it will take time before these issues are fully recognized.

2. Compensatory feeding

Three almost simultaneous papers addressed the issue of compensatory feeding (CF) in *Drosophila* aging experiments. Whereas our data clearly indicated CF (2), two of the leading labs in *Drosophila* aging made the opposite claim (6, 7). The work done since then strongly indicates that CF does indeed occur and that the contradictory

claims were a result of methodological differences. While we used radiolabeling, Mair et al. scored the fraction of flies eating at a given time (6), which does not address food intake, while Min et al. relied on long-term (24 h) recordings using dye labeling (7), which, as discussed above, is uninformative. CF has recently been confirmed using the CAFE (4). It is unlikely that the conflicting claims are due to differences in fly strains or culture conditions, since we have extended our original findings to numerous genotypes, diets and animal enclosures, including a set of conditions identical to the ones used in the Partridge lab (4). While our work establishes that flies are capable of CF, the magnitude of compensation depends on the particular conditions being used. Food intake measurements should therefore be an integral part of any experiment measuring lifespan or introducing dietary changes.

3. Water-dependent and water-independent effects of DR

Our results show conclusively that lifespan extension by classical fly DR paradigms is abolished in the presence of *ad libitum* water (4). Although we reproduced this finding in three different sets of conditions (4), the true test of validity will be independent confirmation by other labs. Much like the issue of CF, the hydration caveat had been raised by the fly aging community (12) and even addressed experimentally (13). Ironically, here too the findings were contradictory, with Bass et al. seeing no effect of water supplementation. Once again, the explanation is likely to lie in the details of experimental design. Whereas we provided water in large drops on the vial wall, Bass et al. used a pipette tip, the small surface of which may be

insufficient to ensure access or become occluded by bacterial growth or excreta. The key issue, however, is that Bass et al. made no attempt to confirm water ingestion in their setup. Instead, the researchers simply assumed the source was appropriate, a particularly misleading practice when facing a negative result.

All future studies of fly lifespan should employ conditions in which *ad libitum* water is present or, at least, shown not to affect survival.

4. Demography of DR

Our results show that the immediate and sustained reversion of fly mortality by classical DR (14) is mediated by hydration (chapter 5). The implications are twofold. First, studies based on the original claim currently under way in higher organisms should be reevaluated in light of the new data. Our findings predict that midlife instigated DR will decelerate the rise in death risk in mammals, but not rescue it to the level of chronically exposed animals. Second, our observations indicate that DR acts by slowing the accumulation of age-related damage. It will be of great interest to identify the molecular source of this progressive deterioration. Obvious candidates include free radicals and DNA/protein mutations (15).

Finally, our experiments reveal that DR and temperature have a phenomenologically similar effect on mortality rate, raising the possibility that the two interventions share common mechanisms. This could be addressed by (i) asking if DR

and temperature have an additive effect and (ii) asking if DR-insensitive mutants also fail to respond to the temperature shift paradigm.

5. Pending issues in *Drosophila* DR

The fact that lifespan extension by classical DR paradigms is mediated by hydration and thus may represent an artifact poses a number of questions. Much like the demography issue, all findings based on water-dependent paradigms are now of dubious relevance to understand mammalian DR. For example, fly DR has been claimed to have no effect on metabolic rate, free radical production or mitochondrial density (16–18). It is also thought not to depend on reproductive activity (19). Its effects on genome-wide transcript profiles have been analyzed (20). And several mutants are thought to mediate its lifespan extension (21, 22). All these questions will have to be reevaluated in water-independent paradigms.

6. Calories vs ratio

The dietary mechanisms of DR have been a long-standing mystery in aging research. In rodents, calories were historically believed to mediate the effect of food restriction—hence the term Caloric Restriction commonly used in mammals. This belief has been challenged both in rodents (23) and flies (6, 10). Although it has been claimed that yeast, not sugar, mediates the DR effect in *Drosophila* (24, 6), these studies did not convincingly address food intake and thus could not determine the

volume of each component that was actually ingested, precluding an unambiguous interpretation. On the other hand, a recent study has produced a complete and careful analysis of the effect of nutrients on lifespan (10). The data show that both protein and carbohydrate intake play a role. Notably, increasing carbohydrate intake was always beneficial for longevity within the ranges tested. The authors suggest that the key factor is the ratio between the two nutrients (with 1:16 protein:carbohydrate being ideal), whereas calories themselves have no effect. Since rodents live longer when fed smaller volumes of a given diet (25), i.e., maintaining a constant nutrient ratio, it is possible that the effect of nutrients on lifespan is species specific.

These findings challenge the very designation of DR; the idea that simply eating less is optimal for lifespan is simplistic and obsolete. Dietary Modulation may be a more accurate term.

7. The future of fly aging

If we are to ensure that the next chapter of fly aging is more productive and less riddled with experimental problems than the past, the basic setup used to measure lifespan must be reviewed. The conditions used by Lee et al. (10) are probably the most rigorous to date, although they are associated with unusually short lifespans, possibly due to the small tubes used to age the animals, the small egg-laying surfaces or low humidity. One alternative would be to use regular fly vials containing an agar surface on the bottom—for humidity and egg laying—and capillaries with food inserted through the top. Flies could be housed in groups (less work-intensive) or

individually (more precise feeding measurements). In the latter case, multi-well culture plates could also be used as enclosures.

Eventually, it would be desirable to depart from the food dilution method of DR. In rodent studies, animals are housed individually and fed predetermined volumes of food. This should, in principle, be feasible in flies, using the CAFE. A more ambitious setup would include automated, electronic control of food delivery to individual chambers, perhaps using microfluidics technology.

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Final Remarks

6 years of graduate school have taught me more lessons than I could possibly hope to fit in these pages, but here are the top 10 things I wish I knew before I started. I learned them the hard way. I made all of these mistakes, some more than once, and hopefully, for the last time.

1. Go big or go home

I am only truly motivated if the project at hand has the potential to change a field (or start a new one). I would rather fail at something big than succeed at something small. I should know, I have done both.

2. Be realistic

A big goal is useless if you do not have the means to achieve it. I spent a long time looking for a molecule I did not have the tools to find. Luckily, another group (who did have the tools!) found it and I moved on. Make sure you are pursuing a project, not a dream. If it is a dream, at least make sure it is YOUR dream.

3. Be original

Seymour's favorite piece of advice. Carving your own path is a difficult and lonely task, but it is the only one that makes sense. Living in fear of being scooped and rushing to publish the next obvious experiment a week before another lab is a near-useless contribution.

4. Trust no one

Intellectually, not morally. People generally mean well, but they just do not think hard enough if it is not their time and career on the line. It is human nature. Listen to everyone, but make your own decisions. Take full responsibility for what you do (and do not do).

5. Do the important experiment first

Once I attended a seminar on scientific conduct. The speaker said we should mercilessly assail our own hypothesis and try our best to falsify it. Sydney Brenner, who was sitting next to me, leaned over and whispered "Everybody talks about this, but nobody does it!" Well, maybe we should. At times, I was guilty of not doing the deal-breaker experiments earlier. To spend time on beating-around-the-bush characterization when there is an experiment that can demolish your project is pure denial.

6. Be unbiased

Another Brenner truth. Everybody talks about it, (almost) nobody does it. Falling in love with a project or a technique instead of a problem or the search for its solution; seeing your discoveries and papers as your "babies"; finding the flaw in everybody's work except your own. All death sentences for a scientist.

7. Follow your passion

Research is largely about self-motivation. It is impossible to put in the necessary time and effort if you are not fascinated with your work.

8. Be persistent, not stubborn

Perseverance is one the most lauded qualities in research. And understandably so. But when persistence becomes obstinacy, you have a problem. If everybody tells you you are wrong, you should at least consider that possibility. In the words of Mark Twain, “It ain’t what you don’t know that gets you into trouble. It’s what you know for sure that just ain’t so.” Indefinitely insisting on an approach that simply refuses to work is not tenacious, it is stupid. Sometimes, quitting is the bravest thing to do.

9. Complexity should neither be sought nor avoided

Academics live their lives above their necks. In a world where intelligence is the currency, people tend to show it off. The more complex and ingenious the idea, the less likely it is to work. Scientific problems are hard enough to tackle as it is. As Seymour would say, keep it simple, stupid.

10. Associate with the right people

This is a big one. Nobody lives in a vacuum, no matter how good or bad you are, you are influenced by your peers. Some people are negative, lazy, talentless or all of the above. Avoid them like the plague. Everything that ever worked for me was the result of being surrounded with productive people whose priority was to do good work.

Appendix

Reactive Oxygen Species Regulate Female Sexual Behavior

Gil B. Carvalho, Shlomo de Leon, William W. Ja, Marisol Madrigal, Seymour Benzer and David J. Anderson

Introduction

Drosophila provides a valuable model to understand the molecular and neuronal underpinnings of animal behavior. One of the most remarkable behavioral phenomena in fruit flies is the female post-mating response (PMR), a dramatic behavioral and metabolic switch including stimulated ovulation and oviposition, lowered sexual receptivity, elevated food intake, induced immune function and shortened lifespan (1–5). The PMR is triggered by the sex peptide (SP), produced in the male accessory gland and transferred in the seminal fluid during coitus (1). How does this small peptide exert such dramatic and pleiotropic effects in females? SP acts by binding to and activating its receptor, SPR, present on the membrane of *fruitless*-expressing neurons (6). The signal originating from SPR activation is intracellularly transduced by the cAMP pathway (6, 7) and requires a subset of *apterous*-expressing ventral nerve cord neurons (8), but the molecular mechanisms of the PMR remain largely mysterious.

Reactive oxygen species (ROS) resulting from metabolism or exposure to extrinsic oxidants have both physiological and pathological effects. Although classically thought to be a pernicious metabolic by-product leading mainly to oxidative stress, age-related disease and senescence (9, 10), ROS have recently been ascribed a critical role in such diverse and fundamental processes as immunity, cell cycle and apoptosis (10). Additionally, ROS have been implicated in reproductive function across phyla. Namely, ROS control germline development in *C. elegans* (11), ovarian muscle contraction in *Drosophila* (12) and sexual behavior in rats (13), and

have been linked to oocyte maturation, egg implantation, labor and sterility in humans (14).

Results and Discussion

We asked if ROS play a regulatory role in *Drosophila* sexual behavior. Exposure to high oxygen levels induces ROS accumulation (15) and oxidative protein damage (16). Hyperoxia exposure significantly induced egg laying in mature, virgin females of two wild-type strains (figure 1A and supplementary figure 1). Despite strain-specific egg laying patterns, hyperoxia clearly stimulated fecundity in both cases. Direct administration of the ROS hydrogen peroxide also induced virgin fecundity (figure 1B), supporting the view that the effect of hyperoxia is mediated through elevated ROS levels. Interestingly, the superoxide anion generator paraquat had no effect (data not shown), suggesting that sexual behavior may be specifically controlled by H_2O_2 or its by-products (10), rather than by ROS species in general.

Hyperoxia also abolished virgin sexual receptivity (figure 1C). Direct observation of male-female pairs revealed that control males spent less time courting O_2-exposed females (figure 1D). Additionally, these virgins were more prone to extrude their ovipositor in response to male advances (figure 1E), a behavior typical of mated females (17). These results indicate that the lower receptivity of hyperoxia-treated virgins is due to both (i) reduced female attractiveness leading to reduced courtship and (ii) active female rejection of the type normally seen in mated females. Together, these results show that hyperoxia exposure triggers a behavioral switch reminiscent of the PMR.

To rule out that our observations are a non-specific effect of the environmental interventions, we tested a hypomorphic mutant in subunit B of succinate dehydrogenase (*sdhB*), which accumulates elevated levels of H_2O_2 when compared to a wild-type strain (18). Virgin *sdhB* females showed induced egg laying (figure 2A). This behavior cannot simply be explained by morbidity-induced egg dumping, since the mutant maintained a consistently elevated rate of oviposition for at least several weeks (supplentary figure 2), suggesting that egg production itself may be induced by the mutation. In support of this view, histological analysis of *sdhB* animals revealed a pattern of stimulated oogenesis (figure 2D) that closely resembles the changes seen in mated females (1). Oocyte development consists of 14 stages. The energetically costly process of yolk deposition known as vitellogenesis starts at stages 8 and 9 and becomes more marked at stage 10. In unmated females, a checkpoint ensures that most oocytes accumulate at stages 8 and 9. Those that do progress to stages 10 and beyond tend to accumulate at stage 14, i.e., mature eggs, due to the low egg-laying rate of virgins. Mating, via the action of SP, releases the oogenesis checkpoint (inducing the formation of stage 10 oocytes) and induces egg laying (reducing the number of retained stage 14 oocytes relative to virgins) (1). Our results show that the *sdhB* mutation has a similar effect on oogenesis (figure 2D). Similarly to oxygen-exposed virgins, *sdhB* animals also showed reduced sexual receptivity (figure 2B). Strikingly, male courtship was not affected by the *sdhB* mutation (figure 2C), demonstrating that a ROS-inducing manipulation can lower female receptivity without affecting attractiveness. In conclusion, we have found that environmental, pharmacological and

genetic interventions known to increase ROS levels induce a PMR-like behavioral switch in virgin females.

We next asked whether ROS act in a particular tissue to control sexual behaviors. We targeted the downregulation of *sdhB* to distinct tissues using RNAi. Knockdown of *sdhB* in the nervous system induced virgin egg laying (figure 2E). This effect was specific, because downregulating the gene in a number of other tissues—including glial cells, muscle, fat, the eye, the wing and different parts of the ovary or intestine—was ineffective (data not shown). Although it is formally possible that ROS produced in neurons act elsewhere, this is made unlikely by the tissue specificity of *sdhB* knockdown – if neurons acted merely as a ROS source, one might particularly expect glial cells to be as effective. A subsequent screen of neuronal subpopulations revealed that expression of the double-stranded RNA in cholinergic neurons is sufficient to recapitulate the phenotype (figure 2F), whereas 16 other GAL4 drivers – including, but not limited to, those driving expression in serotonergic, dopaminergic, tyramine/octapaminergic, GABAergic and Fruitless-expressing neurons – had no effect (data not shown). ROS may thus act in the nervous system and cholinergic neurons in particular, to regulate virgin egg laying.

If hyperoxia indeed acts via ROS, then antioxidant agents should counter the effect of oxygen exposure. Overexpression of the mitochondrial form of the antioxidant enzyme superoxide dismutase (mSOD), but not of another five antioxidants, including the cytoplasmic form of SOD (cSOD), strongly rescued the egg laying of virgins exposed to oxygen (figure 3A and data not shown). Furthermore,

driving the mSOD transgene specifically in the nervous system was sufficient to exert a similar effect (figure 3B), supporting the notion that ROS act in neurons to affect fecundity. The antioxidant drug allopurinol (19) also countered the elevation of egg laying typically seen in hyperoxia (figure 3C). This effect was not simply a result of feeding inhibition (supplementary figure 3). On the other hand, neither mSOD overexpression nor allopurinol rescued receptivity in oxygen-exposed females (figure 3D, E and supplementary figure 4). Thus, oxygen may control egg laying via ROS, and receptivity via an unrelated mechanism, although we cannot exclude the possibility that the two behaviors respond to different ranges of ROS, such that the antioxidant treatments used here reduce ROS levels enough to affect one but not the other. Regardless, our observations effectively uncouple the two behaviors and show that, like SP (20), oxygen exposure can trigger sexual rejection in females irrespective of egg laying. To continue to address the epistasis between the various effects of ROS exposure, we tested flies with defects of increasing magnitude at four levels of the reproductive process—$T\beta h^{nM18}$ animals produce normal eggs but are unable to ovulate (21), D1 and D2 alleles of *ovo* arrest oogenesis mainly before stages 4 and 6, respectively (22), whereas $tudor^{1}$ flies lack a germline entirely (23). Hyperoxia reduced the receptivity of $T\beta h^{nM18}$ females indistinguishably from its heterozygous control (figure 4A). While both *ovo* alleles were normally receptive in normoxic conditions and significantly less so in hyperoxia, the magnitude of the effect was diminished relative to the wild type (figure 4B; cf. figures 1C and 4A). A similar situation was observed with $tudor^{1}$ (figure 4C). Collectively, these data suggest that ROS inhibit female receptivity in two discrete ways, one oogenesis-dependent and one

oogenesis-independent, with additive effects, while oviposition behavior has either a small or no influence on receptivity (figure 4D).

We next asked if downregulation of antioxidants in virgins is sufficient to trigger behaviors typically seen in mated females. Preliminary data indicated that ubiquitous or nervous system-specific knockdown of cSOD, but not of mSOD or catalase, significantly stimulated egg laying (supplementary figure 5A, B and data not shown). However, when the experiment was repeated after extensive outcrossing of the transgenic lines, no effect was seen (supplementary figure 5C). The preliminary result may be attributable to genetic background differences, rather than the downregulation of cSOD per se. Alternatively, cSOD may play a lesser role in the background used to isogenize the lines—e.g., due to redundant mechanisms. More work will be required to distinguish between these possibilities.

The fact that high ROS levels induce behavioral changes reminiscent of the PMR suggests that the PMR is mediated by ROS. This could occur at three levels: (i) by affecting the availability or characteristics of SP, (ii) by facilitating the SP-SPR interaction or (iii) by acting downstream of SPR after its activation, e.g., by intracellular signal transduction. Hyperoxia stimulated fecundity regardless of the presence of SPR (figure 5A), indicating that ROS act either downstream or in parallel to SPR. We therefore asked if the PMR requires high ROS levels. Ubiquitous overexpression of mSOD mild but significantly rescued fecundity and ovulation following SP administration (figure 5B and supplementary figure 6), with no effect on receptivity (figure 5C). Allopurinol also reduced egg-laying rate upon SP

administration (figure 5D) or mating (figure 5F), but its impact on receptivity was variable. The drug mild but significantly rescued receptivity after SP administration, but had no effect after mating (figure 5E, G). The reason for this discrepancy is unclear. We cannot rule out a ROS-independent effect of allopurinol on the SP-expressing transgenic strain, e.g., an interference with the expression or secretion of SP in this line. Alternatively, SP secreted into the hemolymph in the transgenic line may interact with SPR in target tissues that are normally not accessible to SP transferred during copulation and stored in the female reproductive tract (24, 25). If so, the conflicting results could be a product of tissue-specific drug availability or ROS action.

In humans, the cellular response to oxygen levels is controlled by the transcription factor hipoxia-inducible factor 1 (HIF-1), a heterodimer composed of subunits α and β. Under well-oxygenated conditions, HIF-1α undergoes post-translational modifications and proteolytic degradation, whereas under hypoxia it binds the β subunit, translocates to the nucleus and activates the transcription of key response genes (26). In addition to O_2, HIF-1α also responds to ROS levels (26). We asked if the *Drosophila* HIF-1α homolog, Sima (27), mediates the effect of ROS on sexual behaviors. Since high oxygen levels lead to HIF-1α degradation, one might expect overexpression of Sima to counter the effect of hyperoxia. Ubiquitous Sima overexpression led to embryonic lethality, but nervous system–specific overexpression generated healthy adults with attenuated fecundity upon exposure to O_2 (figure 6). It will be interesting to extend the analysis of the role of Sima in controlling reproductive behaviors, both in the context of hyperoxia and the PMR.

Experimental Procedures

***Drosophila* strains and culture conditions.** Flies were raised and aged on Lewis medium (28) and sexed under light CO_2 anesthesia. In all experiments, flies were kept in polystyrene vials (Genesee Scientific) plugged with cotton stoppers. Trials were conducted at 25°C and controlled humidity on a 12 h:12 h light:dark cycle. The default wild-type strain is Canton-S. The stock *sdhB*-IR was obtained from the Japanese National Institute of Genetics stock collection. The transgenic strains used to overexpress or downregulate the various antioxidant enzymes were generously provided by F. Missirlis, J. Philips, R. Mockett, U. Banerjee and W. J. Lee, $T\beta h^{nM18}$ by M. Heisenberg, Fru-GAL4 and SPR-IR by B. Dickson and *yp*1-SP (Yp1-hsp70-SPgene) by E. Kubli. All other stocks were obtained from the Bloomington Stock Center. Transgenic strains were outcrossed at least 6 times into our *w1118* strain. Control crosses, denoted with a (+), consist of a copy of the transgene in trans to UAS-GFP-IR (B. Baker) to control for *white* expression levels.

Hyperoxia. Fly vials were kept in a C-474 culture chamber (Biospherix, Redfield, NY). Oxygen inflow from industrial cylinders was regulated by a ProOx 110 oxygen sensor/controller (Biospherix) calibrated before each experiment. An average oxygen concentration of 85% was reached inside the chamber. Temperature/humidity/light were set to match normoxic conditions. Virgin females were exposed to hyperoxia upon reaching maturity (3–5 days of age).

Food additives. Lewis medium was prepared without cornmeal and drugs added when food cooled to ~65°C. All compounds were purchased from Sigma-Aldrich. Flies were

switched from normal to supplemented food upon reaching maturity (for H_2O_2 and paraquat) or from eclosion (for allopurinol).

Behavioral assays

Egg laying. Except where otherwise noted, virgin females were kept at a density of 5 animals per vial. Fresh food was provided and eggs scored daily, starting at eclosion (for genetic manipulations) or maturity (for acute treatment with hyperoxia or drugs).

Receptivity, courtship and ovipositor extrusion. Individual females were transferred without anesthesia (by aspiration) to circular mating chambers (1 cm diameter, ~0.6 cm height) containing a wet filter paper circle, paired with 1 virgin, control (Canton-S) male and observed for 1 h. Successful copulation was scored. For courtship and ovipositor extrusion assays, male-female pairs were filmed for 10 min and analyzed individually.

Ovulation. Flies under light CO_2 anesthesia were individually assayed for the presence of an egg in the uterus by gently squeezing the tip of the abdomen with forceps.

Feeding rate. Intake was determined as described (2).

Ovary dissection. The ovaries of mature, CO_2 anesthetized virgins were dissected with forceps in 1x PBS, stained with DAPI for 5 min, transferred onto a glass slide and covered with mounting medium. Ovarioles were carefully teased apart and the preparation covered with a glass cover slip. Oocytes were staged under light microscopy according to (29).

SP injection. ~200 nL of 240 μ*M* SP in ddH_2O was injected, with a glass microcapillary, into the thorax of CO_2-anesthetized, mature virgin females. Flies were transferred to fresh food vials and allowed to recover.

Statistical analysis. For egg laying as a function of time, two-way ANOVA was applied.

For all other data, 2-tailed student's t-test and one-way ANOVA (for >2 groups) were applied.

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Figure legends

Figure 1. ROS affect female sexual behavior. (A, B) Hyperoxia exposure (A) or H_2O_2-laced food (B) stimulate egg laying in virgins females. n = 5–10 replicates of 5 animals per condition. (C) Hyperoxia abolishes virgin sexual receptivity. Mature virgins exposed to either normoxia or hyperoxia for 48 h were housed, in normoxic conditions, with control males for 2 h and allowed to mate. n = 2 replicates of 10 animals per condition. (D) Hyperoxia-exposed females elicit lower male courtship index (defined as the percentage of time spent courting by the male). n = 12 animals per condition. (E) Hyperoxia exposure induces ovipositor extrusion in virgins. Extrusion index is defined as percentage of time spent with an extruded ovipositor over total time in which the female was courted. n = 7-9 animals per condition. Error bars represent s.d. in (C), s.e.m. in all other panels. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 2. *sdhB* affects female sexual behavior. (A) *sdhB* hypomorphs resulting from imprecise excision of a transposon exhibit increased egg laying when compared to precise excision controls. (B) *sdhB* regulates oogenesis. Mutants show an increase in oocytes in vitellogenic stage 9 and a decrease in mature, stage 14, eggs. (C) *sdhB* animals show reduced sexual receptivity to wild-type males. (D) *sdhB* mutants elicit normal levels of male courtship. (E, F) Neuronal SdhB levels are critical for the control of sexual behavior. Tissue-specific downregulation of *sdhB* via RNAi either throughout the nervous system (E) or specifically in cholinergic neurons (F) is sufficient to induce virgin fecundity. n = 3 animals per genotype in (B), 10 replicates

of 3 animals in (C–D) and 10 replicates of 5 flies in all other panels. Error bars represent s.e.m. n.s. (not significant), P>0.05; **, P<0.01; ***, P<0.001.

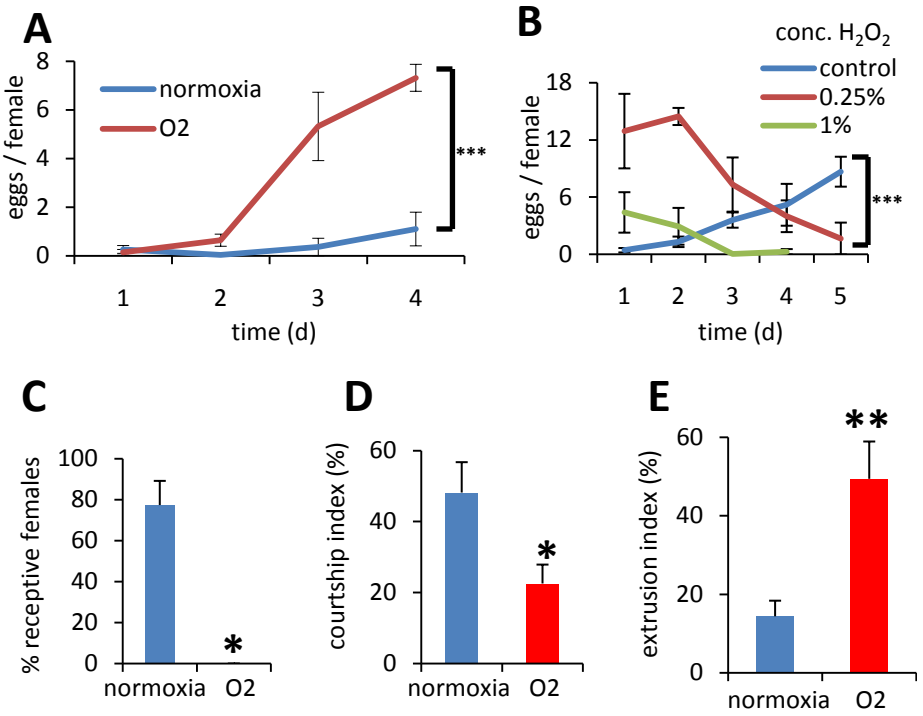
Figure 3. Antioxidants counter the effect of oxygen on fecundity. (A, B) Ubiquitous (A) or neuron-specific (B) mSOD overexpression suppresses the induction of egg laying seen in hyperoxia. (C) The antioxidant drug allopurinol partially suppresses the effect of hyperoxia on egg laying. 0, 5 or 20 mM allopurinol were added to the food medium. (D, E) Neither mSOD overexpression (D) nor allopurinol feeding (E) affect the low receptivity of O_2-exposed virgins. In (A-C), n = 5 replicates of 5 flies per condition and error bars represent s.e.m. In (D–E), n = 2 replicates of 8–10 flies and error bars represent s.d. n.s., P>0.05; **, P<0.01; ***, P<0.001.

Figure 4. Role of oviposition and oogenesis in the ROS-induced behavioral switch. The receptivity of $T\beta h^{nM18}$, ovo^{D1}, ovo^{D2} and $tudor^{1}$ mutants was determined before and after hyperoxia treatment. While $T\beta h^{nM18}$ females are statistically indistinguishable from their heterozygous controls (A), the *ovo* (B) and *tudor* (C) alleles partially restore virgin receptivity under high O_2 levels. (D) Model for the epistatic relationship between ROS-controlled behaviors. ROS exposure induces both oogenesis and oviposition in virgin females. Sexual receptivity is inhibited both by oogenesis and by ROS directly. n = 4–8 replicates of 4–15 animals per condition. Error bars represent s.e.m. n.s., P>0.05; *, P<0.05; ***, P<0.001.

Figure 5. Regulation of female sexual behavior by SP and ROS. (A) Stimulation of fecundity by O_2 does not require SPR. SPR knockdown virgin females – bearing both UAS-SPR-IR and the *fru*-GAL4 driver (fru) – (right panel) show normal egg-laying

induction when exposed to O_2, as compared to the driver control (left panel). n = 5 replicates of 5 animals per condition/genotype. (B, C) Ubiquitous overexpression of mSOD partially suppresses egg laying (B), but not receptivity (C) after SP administration (by injection). n = 3–4 replicates of 5–13 animals per genotype. (D, E) Allopurinol feeding partially rescues both egg laying (D) and receptivity (E) upon ectopic expression of SP. n = 3–6 replicates of 5 (D) or 10 (E) animals per condition. (F, G) Allopurinol feeding partially suppresses egg laying (F), but not receptivity (G) 6 days after mating. n = 2–3 replicates of 5 animals for (F), 3–6 replicates of 10 animals for (G). Error bars represent s.d. in (F), s.e.m. in all other panels. n.s., $P>0.05$; *, $P<0.05$; ***, $P<0.001$.

Figure 6. Overexpression of Sima in neurons attenuates the effect of O_2 on virgin fecundity. n = 5–6 replicates of 5 animals per condition. Error bars represent s.e.m.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extrusion index (%)
normoxia
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Figure 1

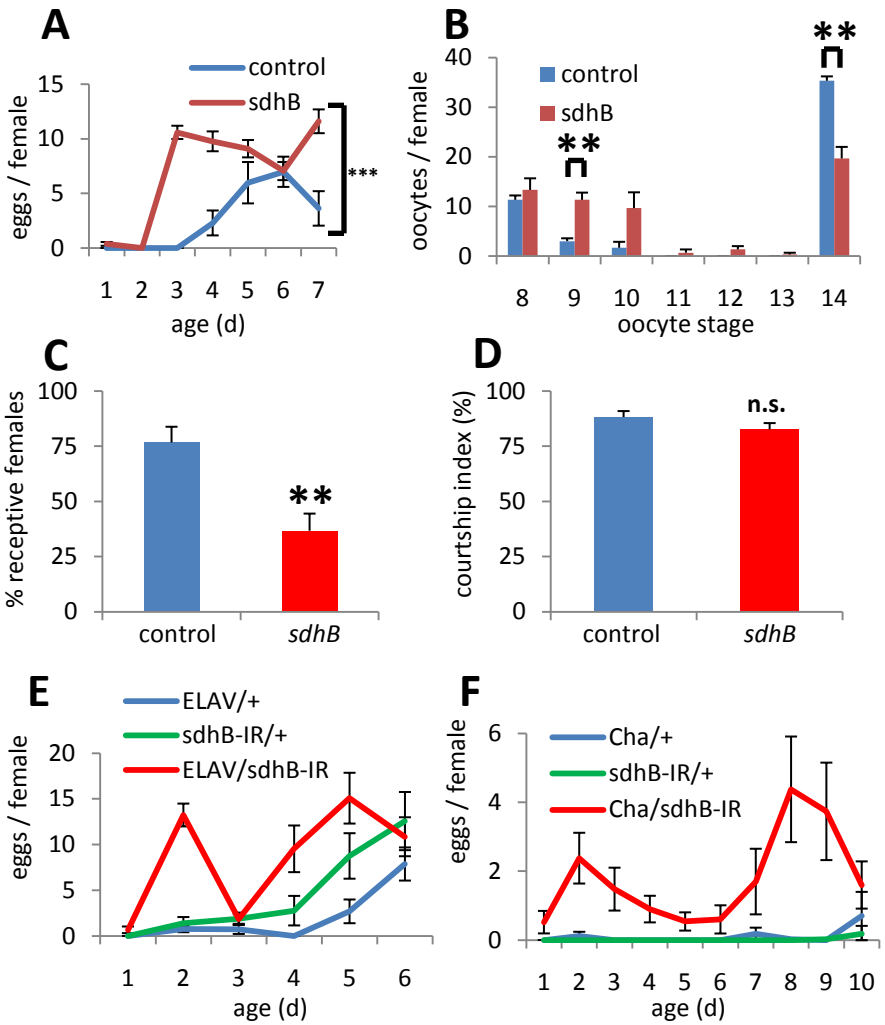
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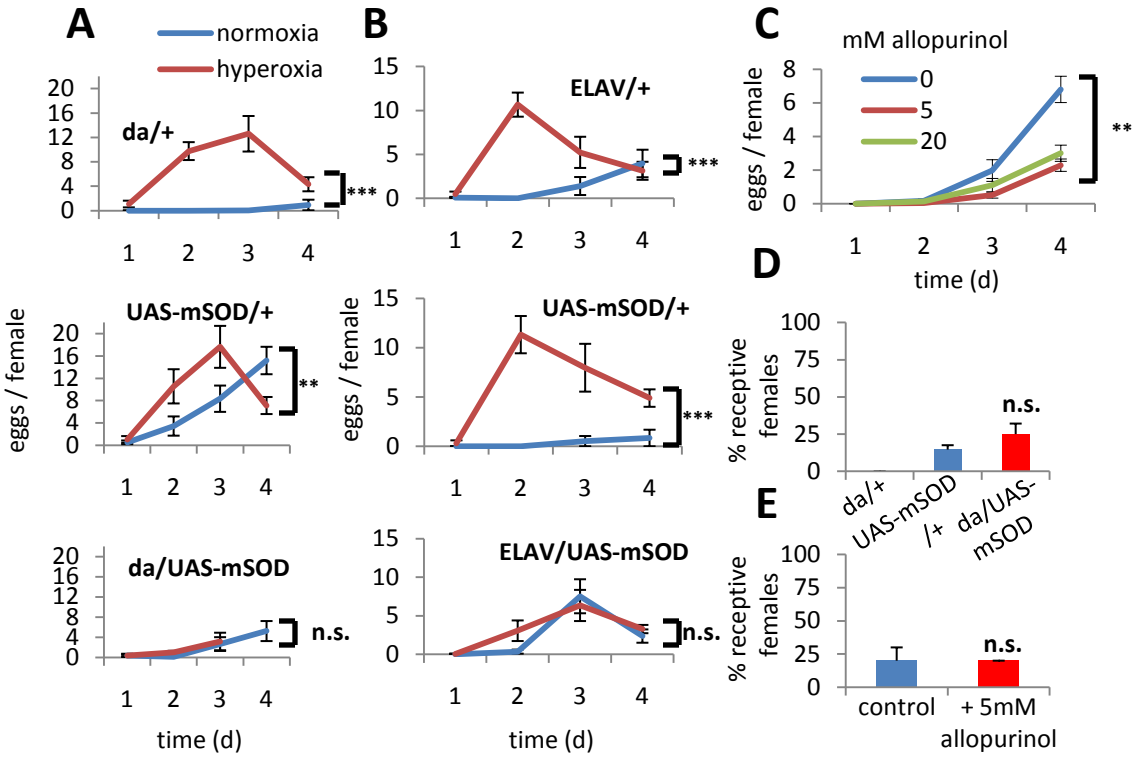
Figure 2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UAS-mSOD/+
***
ELAV/UAS-mSOD
n.s.
time (d)
C
mM allopurinol
0
5
20
**
eggs / female
time (d)
D
% receptive females
n.s.
da/+
UAS-mSOD /+
da/UAS-mSOD
E
% receptive females
n.s.
control
+ 5mM allopurinol

Figure 3

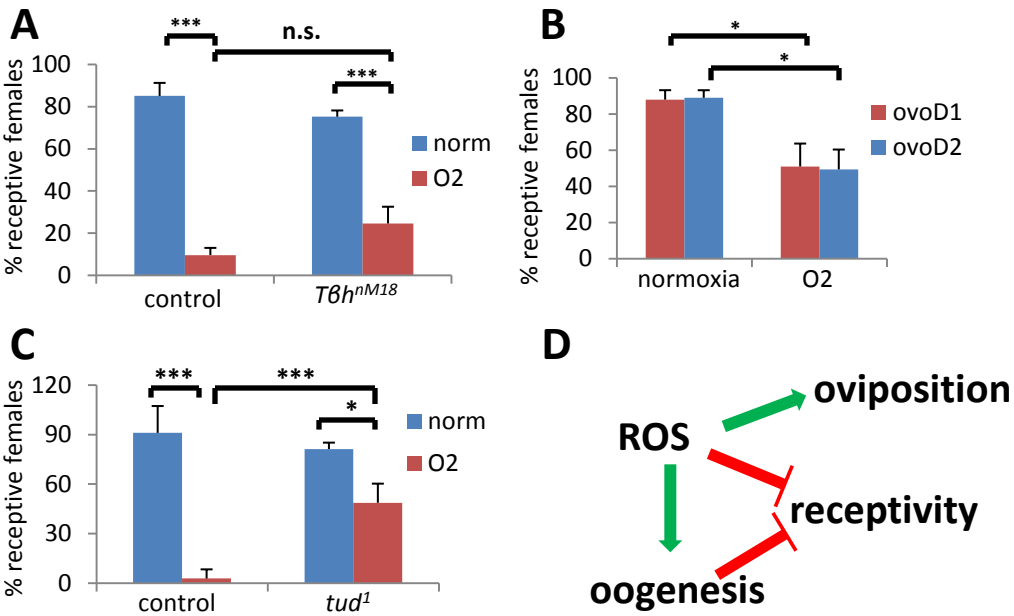

A
***
n.s.
***
% receptive females
100
80
60
40
20
0
norm
O2
control
Tβh^nM18
B
*
*
% receptive females
100
80
60
40
20
0
ovoD1
ovoD2
normoxia
O2
C
***
***
*
% receptive females
120
90
60
30
0
norm
O2
control
tud1
D
ROS
oviposition
receptivity
oogenesis

Figure 4

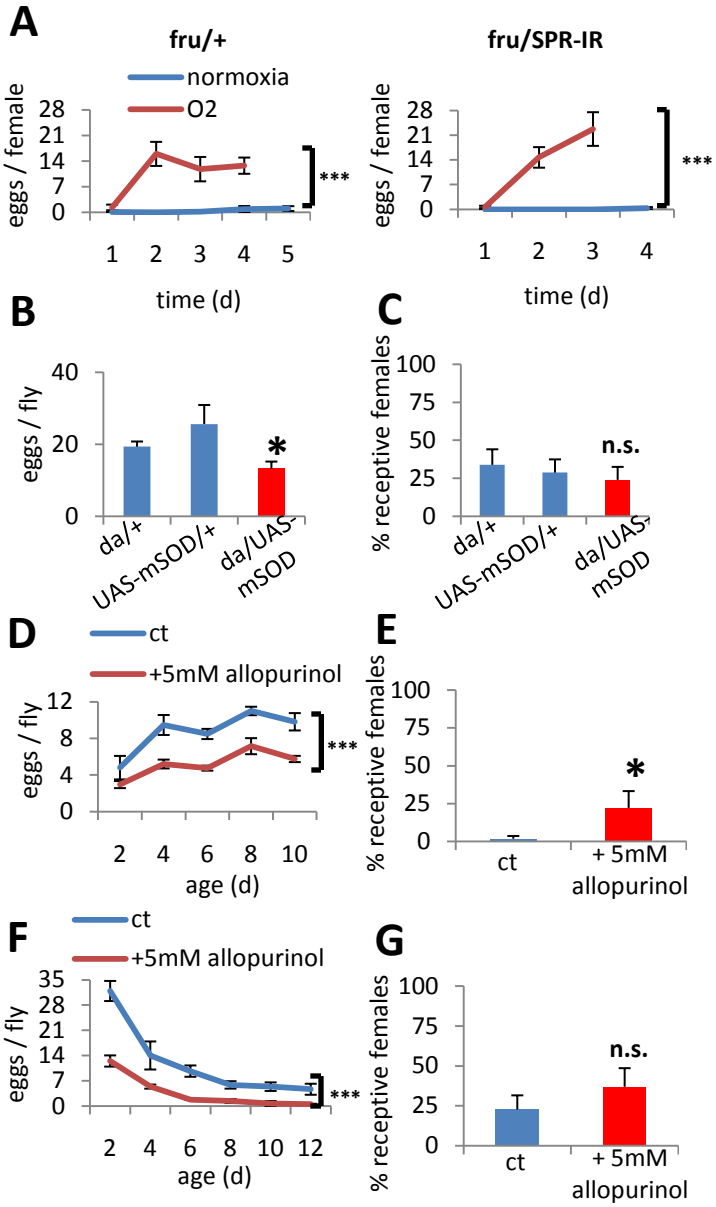

A
fru/+
fru/SPR-IR
normoxia
O2
eggs / female
28
21
14
7
0
1 2 3 4 5
time (d)
1 2 3 4
***
B
eggs / fly
40
20
0
da/+
UAS-mSOD/+
da/UAS-mSOD
*
C
% receptive females
100
75
50
25
0
n.s.
D
ct
+5mM allopurinol
12
8
4
2 4 6 8 10
age (d)
E
ct
+ 5mM allopurinol
F
35
28
21
14
2 4 6 8 10 12
G

Figure 5

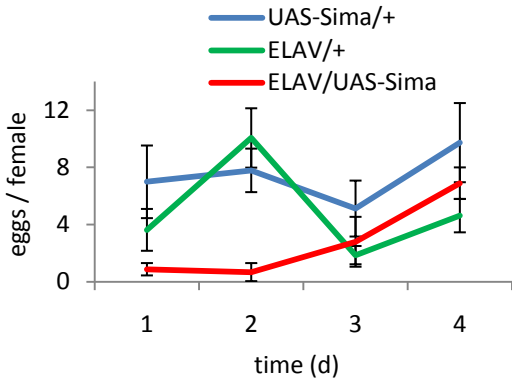


Figure 6

Supporting Information

Supplemental Figure 1**.** Hyperoxia exposure stimulates egg laying in wild type Oregon R virgins. n = 10 replicates of 5 animals per condition. Error bars represent s.e.m. ***, P<0.001.

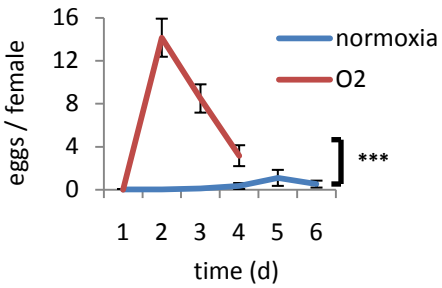
Supplemental Figure 2**.** *sdhB* virgins show a sustained elevation in egg-laying rate up to at least 30 days of age. n = 10 replicates of 5 flies per genotype. Error bars represent s.e.m.

Supplemental Figure 3**.** Addition of allopurinol to food medium does not alter ingestion as assayed by isotope radiolabeling. n = 6–12 replicates of 5 flies per genotype. Error bars represent s.e.m. n.s., P>0.05.

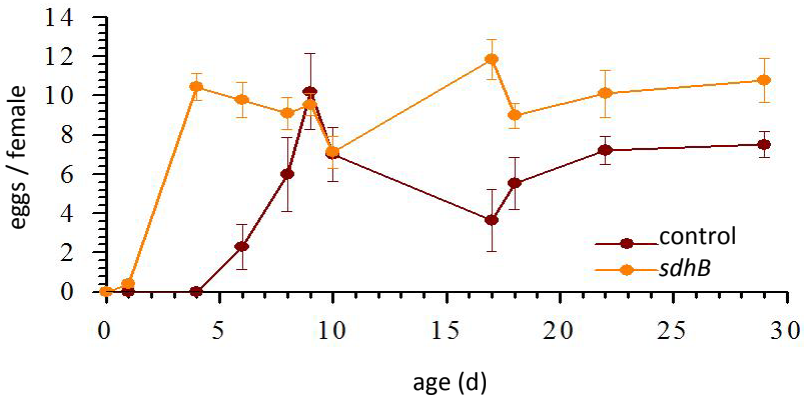
Supplemental Figure 4**.** Allopurinol feeding does not rescue receptivity of virgins exposed to O_2. Females either kept in normoxia or exposed to hyperoxia for 1 or 2 days were tested. n = 2–4 replicates of 10 animals. Error bars represent s.d.

Supplemental Figure 5**.** Antioxidant downregulation and virgin fecundity. Preliminary data show that ubiquitous (A) or nervous system-specific (B) downregulation of cSOD induces egg laying in virgins, whereas after extensive outcrossing (C) neither driver shows an effect, nor does Cha-GAL4, which drives expression in cholinergic neurons. n = 7–9 replicates of 5 animals. Error bars represent s.e.m.

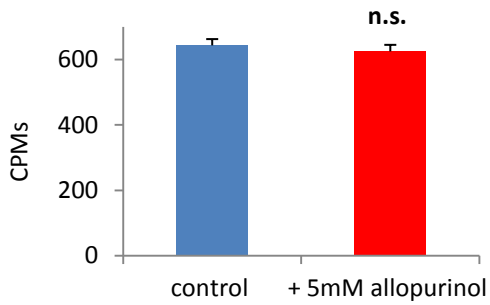
Supplemental Figure 6**.** Ovulation after SP injection is inhibited by ubiquitous overexpression of mSOD. n = 7–8 replicates of 10-30 animals. Error bars represent s.e.m. **, P<0.01.



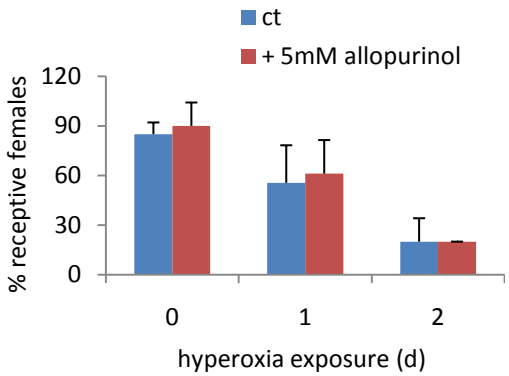
Supplementary Figure 1



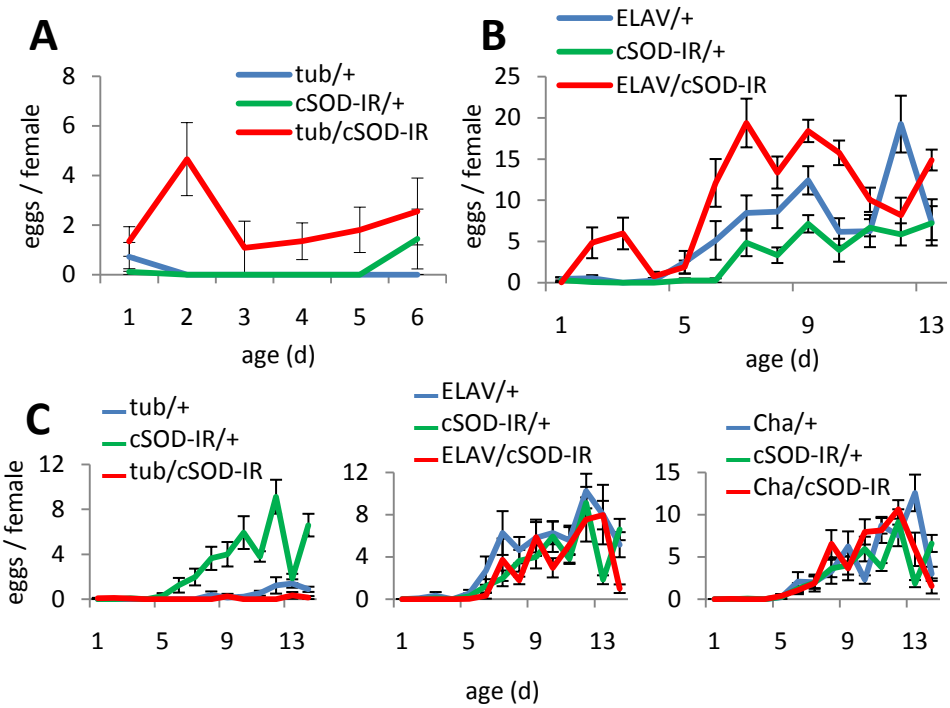
Supplementary Figure 2



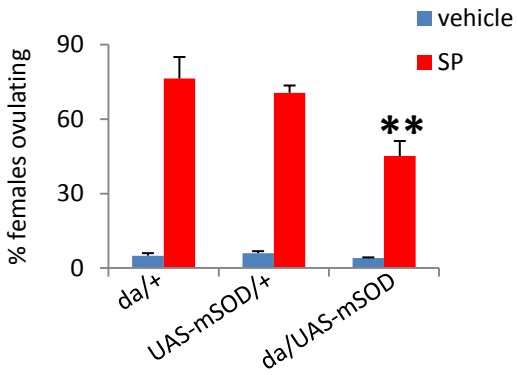
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6