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RESEARCH ARTICLE

Altered regulation of sleep and feeding contributes to starvation resistance in *Drosophila melanogaster*

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ABSTRACT

Animals respond to changes in food availability by adjusting sleep and foraging strategies to optimize their fitness. Wild populations of the fruit fly, *Drosophila melanogaster*, display highly variable levels of starvation resistance that are dependent on geographic location, food availability and evolutionary history. How behaviors that include sleep and feeding vary in *Drosophila* with increased starvation resistance is unclear. We have generated starvation-resistant flies through experimental evolution to investigate the relationship between foraging behaviors and starvation resistance. Outbred populations of *D. melanogaster* were selected for starvation resistance over 60 generations. This selection process resulted in flies with a threefold increase in total lipids that survive up to 18 days without food. We tested starvation-selected (S) flies for sleep and feeding behaviors to determine the effect that selection for starvation resistance has had on foraging behavior. Flies from three replicated starvation-selected populations displayed a dramatic reduction in feeding and prolonged sleep duration compared to fed control (F) populations, suggesting that modified sleep and feeding may contribute to starvation resistance. A prolonged larval developmental period contributes to the elevated energy stores present in starvation-selected flies. By preventing S larvae from feeding longer than F larvae, we were able to reduce energy stores in adult S flies to the levels seen in adult F flies, thus allowing us to control for energy storage levels. However, the reduction of energy stores in S flies fails to generate normal sleep and feeding behavior seen in F flies with similar energy stores. These findings suggest that the behavioral changes observed in S flies are due to genetic regulation of behavior rather than elevated lipid levels. Testing S-F hybrid individuals for both feeding and sleep revealed a lack of correlation between food consumption and sleep duration, indicating further independence in genetic factors underlying the sleep and feeding changes observed in S flies. Taken together, these findings provide evidence that starvation selection results in prolonged sleep and reduced feeding through a mechanism that is independent of elevated energy stores. These findings suggest that changes in both metabolic function and behavior contribute to the increase in starvation resistance seen in flies selected for starvation resistance.

KEY WORDS: *Drosophila*, Experimental evolution, Feeding, Foraging, Sleep

INTRODUCTION

Selection for starvation resistance results in increased energy stores and modified metabolism (L.A.R. and A.G.G., personal communication; Schwasinger-Schmidt et al., 2012). Despite this knowledge, little is known about the behavioral adaptations that facilitate starvation resistance. Both sleep and feeding are regulated by metabolic state and potentially contribute to starvation resistance, suggesting that these behavioral traits are under selective pressure during times when food resources are scarce (Griffith, 2013; McDonald and Keene, 2010). Acute modulation of these behaviors occurs throughout the animal kingdom, and both sleep and foraging strategies appear to be influenced by evolutionary history and ecological niche (Siegel, 2008). Further, starved insects and rodents suppress sleep and increase activity, presumably to initiate food-seeking behavior (Borbély, 1977; Keene et al., 2010; Lee and Park, 2004). Although functional interactions between foraging and sleep have been described, the evolutionary trade-offs between these behaviors has been difficult to test experimentally. Both sleep and feeding are highly variable in *Drosophila*, and naturally occurring polymorphisms have been identified that modulate the interaction between these processes (Kaun et al., 2007; Keene et al., 2010; McDonald and Keene, 2010; Donlea et al., 2012).

Characterization of wild-caught or outbred fly populations is an effective method to examine the relationship between the environment of a population and a naturally occurring trait, such as starvation resistance. Starvation resistance in wild populations of *Drosophila* has been extensively studied across many geographic ranges and is often found to be strongly correlated with lipid or energy content of the flies (Ballard et al., 2008; Marron et al., 2003). An alternative application of wild-caught lines is experimental selection of outbred lines to study the evolutionary basis for, and interactions between, traits. Both experimental evolution and artificial selection have been used to generate flies with dramatically transformed sleep and metabolic function. Selection for starvation resistance resulted in prolonged larval development, elevated energy stores and a slower metabolic rate (Harshman et al., 1999; L.A.R. and A.G.G., personal communication). Here, we examine the trade-off between sleep and foraging behaviors in flies selected for starvation resistance to determine their relationship to metabolite content and contribution to increased starvation resistance.

We have utilized experimental evolution to generate starvation resistant flies (*Drosophila melanogaster*, Meigen 1830) with dramatically enhanced longevity in response to starvation stress. Three replicated lines of starvation resistant flies (S populations) were generated by selecting for survival on a non-caloric agar diet and compared with three replicated populations of fed control flies (F populations). S flies live up to 18 days without food compared with the 2 to 3 days of F flies. This survival is in part due to the S flies having dramatically elevated total lipid levels compared with F flies (L.A.R. and A.G.G., personal communication). The S flies accumulate these lipids during larval development rather than adulthood, implicating larval feeding and metabolism in the elevated lipid stores present in these flies (L.A.R. and A.G.G., personal

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communication). Selection for starvation resistance results in increased energy stores and modified metabolism (L.A.R. and A.G.G., personal communication; Schwasinger-Schmidt et al., 2012), but little is known about the behavioral adaptations that facilitate starvation resistance.

Here, we have performed a detailed analysis of starvation-induced behavior in S flies to examine the relationship between adult foraging-related behaviors and selection for starvation resistance. Surprisingly, S flies have dramatically reduced food intake and prolonged sleep duration as adults, raising the possibility that the foraging behaviors modulating the rate of energy usage are also critical for survival. These findings support the notion that sleep and feeding represent two mutually exclusive behaviors that are highly plastic in accordance with an animal's environment and evolutionary history.

RESULTS

Analysis of starvation resistance using *Drosophila* activity monitors

S flies were selected for starvation resistance over 60–80 generations (Fig. 1A). All three S population replicates (groups S_A, S_B and S_C) had dramatically elevated lipid stores and were visibly larger than fed control populations (F_A, F_B and F_C) (Fig. 1B). Starvation resistance was determined as the amount of time elapsed from placing the fly into tubes containing 1% agar solution until the final activity point. All three S populations survived longer than F population controls (Kaplan–Meier analysis, log-rank method: χ^2=155.5, d.f.=1, P<0.001; Fig. 1C), confirming that selection for starvation resistance increases survival in the absence of food. The three S populations lived up to 18 days, whereas the control flies lived a maximum of 7 days. No significant difference in survival was observed between the three S populations, suggesting that starvation resistance was equally robust in each of the independently selected S population replicates (Kaplan–Meier analysis, log-rank method: χ^2=2.1, d.f.=2, P=0.345; Fig. 1C). Taken together, these results support the notion that experimental selection for starvation resistance dramatically prolongs longevity in the absence of food.

Feeding behavior is dramatically reduced in starvation-resistant adult *Drosophila*

Obesity in *Drosophila* can be induced in the laboratory by manipulating traits, such as decreasing metabolic rate, prolonging larval development, or increasing food consumption (Al-Anzi et al., 2009; Hathiramani et al., 2011; Liu et al., 2012). It has been suggested that larval development is a primary contributor to evolved starvation resistance, which is attributed to significantly elevated lipid levels in newly eclosed adults (L.A.R. and A.G.G., personal communication). However, it is possible that altered foraging strategies, including changes in adult feeding behavior or sleep, contribute to starvation resistance. We assayed the feeding behavior of adult S flies to determine whether increased adult feeding contributes to elevated nutrient stores. Acute feeding can be measured by providing flies with 5% sucrose containing blue dye and then determining spectrophotometric absorbance of individual homogenized flies (Wong et al., 2009). Short-term food intake was determined by placing sated or fasted flies on sucrose medium that was laced with blue dye for 30 min. In adult F flies, food deprivation for 24 h significantly increased feeding compared with fed F flies (*post hoc* test, $t_{55.2}$=–6.1, P<0.001; see also figure legend of Fig. 2A,B), but no difference in food intake was observed between food-deprived and sated S population flies (*post hoc* test, $t_{76.3}$=–1.9, P=0.066; Fig. 2A,B). This suggests that 24 h of fasting is not

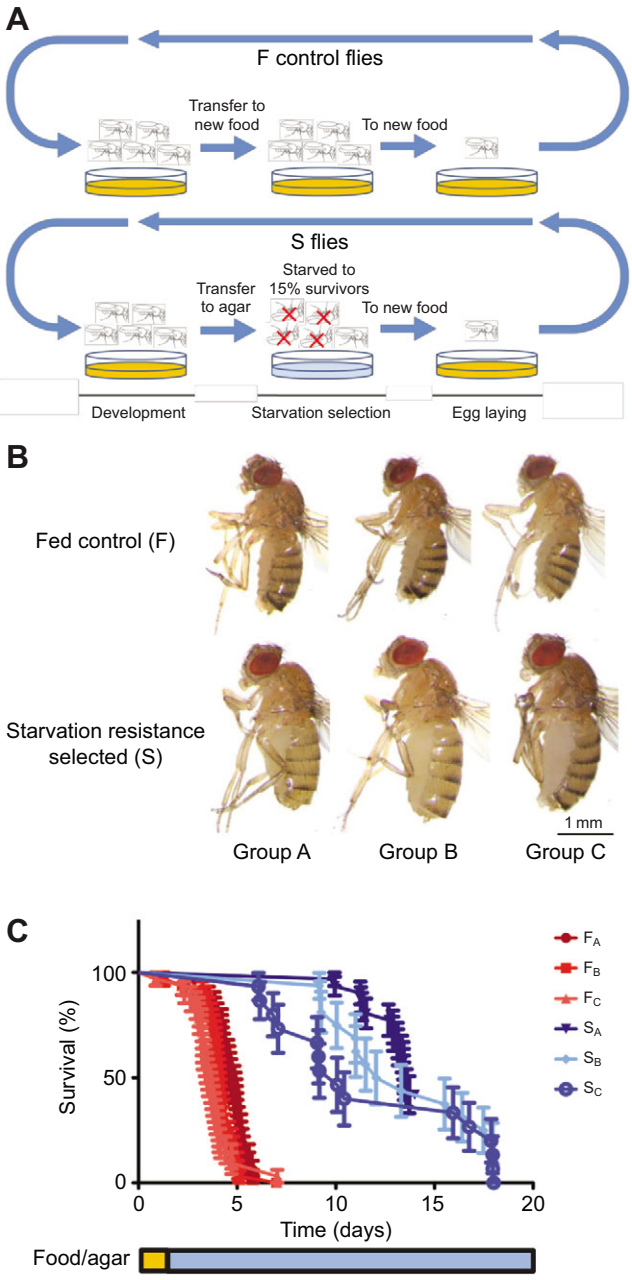

Fig. 1. Generation of starvation-resistant flies. (A) Three outbred fed control (F) populations and three starvation-selected (S) populations were used in these experiments. S populations were maintained on agar until only 15% of the population survived, then were transferred to food to lay eggs. F populations were maintained on food throughout this process. These steps were repeated for >60 generations. (B) All three F populations (top) are visibly smaller than S groups (bottom). (C) Survival of individual flies was measured following transfer of flies onto agar. All S populations survived significantly longer on agar compared with F flies (Kaplan–Meier analysis: χ^2=155.5, d.f.=1, P<0.001 between S and F populations).

sufficient to enhance food intake in flies selected for starvation resistance. Food intake of all three fasted S population group flies was significantly reduced compared with that of fasted F population controls (*post hoc* test with Bonferroni correction: $t_{11.6}$=4.5, $t_{15.9}$=3.7, $t_{15.9}$=3.9, for A, B and C group, respectively, all P<0.01), confirming that feeding in response to food deprivation is reduced in starvation-selected flies. These results indicate the elevated energy stores

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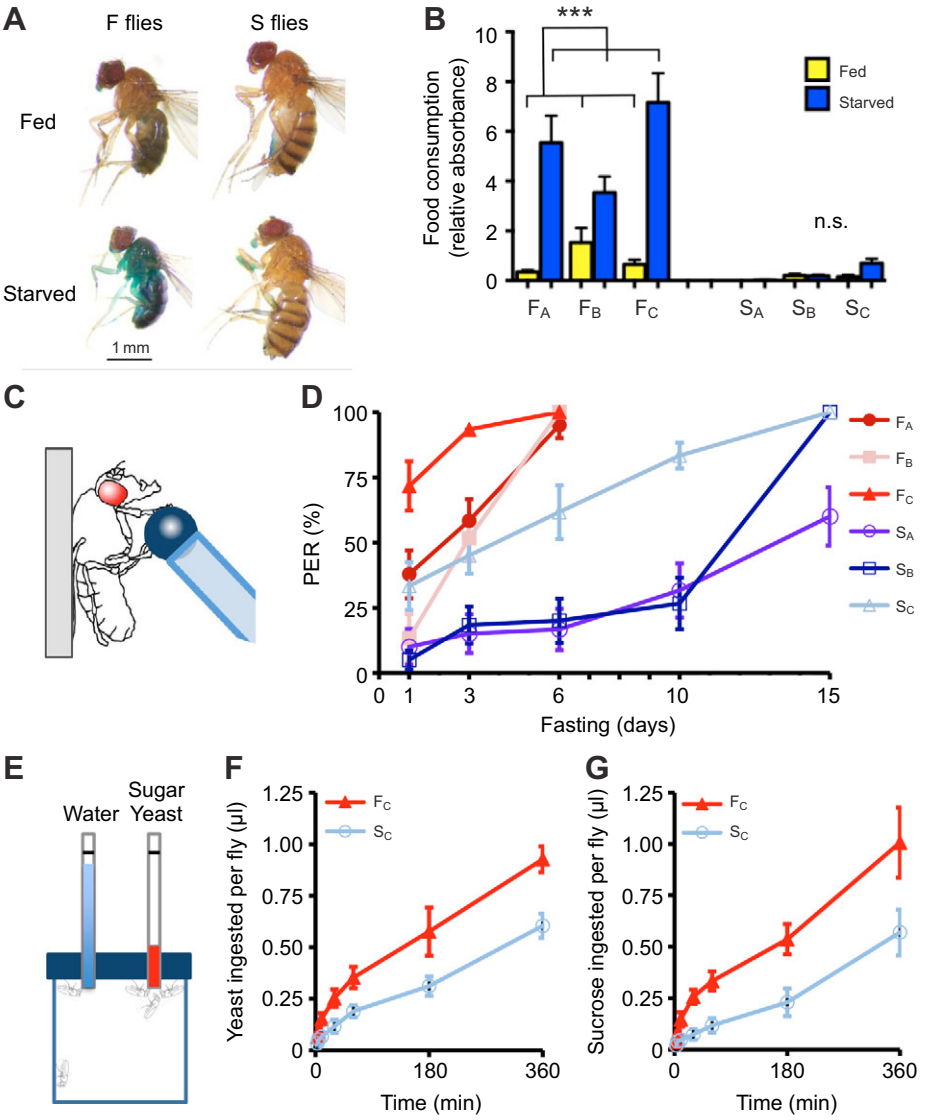

Fig. 2. Fasting-induced feeding is reduced in S populations. (A) Elevated levels of a blue dye are clearly more abundant in the abdomen of 48 h fasted F flies compared with fed controls. Blue dye was not visible in fasted or fed S populations. (B) Spectroscopy based quantification of blue-dye levels revealed a significant increase of food consumption in fasted flies (two-way ANOVA: $F_{1,180}$=44.2, P<0.001, N=92 and 92, fed and starved conditions, respectively) and increase in F populations ($F_{1,180}$=48.1, P<0.001, N=88 and 96, F and S populations, respectively). In addition, there is a significant interaction between food consumption and population ($F_{1,180}$=35.4, P<0.001), indicating different responses to the starvation between S and F populations. The *post hoc* test revealed the fasted F population increased food consumption ($t_{55.2}$=–6.1, P<0.001, N=44 for fed and starved condition), whereas no difference was found for the S population ($t_{76.3}$=–1.9, P=0.066, N=48 for fed and starved conditions). (C) Reflexive feeding was measured by providing flies with sucrose and measuring the PER. (D) The slope of the grouped F populations were significantly steeper than those of the grouped S population [ANCOVA (group×day interaction): $F_{1,474}$=56.9, P<0.001, N=60 for F and S groups], suggesting that PER was significantly higher in equally fasted F flies compared with S populations. (E) Long-term consumption of 150 mmol l^{-1} sucrose and 5% yeast was measured in the CAFÉ assay. (F) The C group F flies consumed significantly more yeast within 10 min and more sucrose within 30 min than C group S flies (P<0.039 and P<0.013, respectively). n.s., not significant, ***P<0.001.

present in starvation-selected flies are not due to greater food consumption during adulthood.

Motivated feeding can be assayed in flies by measuring the proboscis extension reflex (PER) response (Keene and Masek, 2012). An appetitive tastant consisting of 100 mmol l^{-1} sucrose was briefly presented to the feet of a mounted fly and proboscis extension was measured (Fig. 2C). Fasting dramatically increases PER response in flies, suggesting that this behavior reflects internal food drive (Dethier, 1976). Tastants were provided to the feet of a mounted fly and PER in response to sucrose was measured (Fig. 2C). PER was quantified for flies fasted 1, 3, 6, 10 and 15 days prior to testing. The slope of the grouped F populations were significantly steeper than that of the grouped S population [ANCOVA (group×day interaction): $F_{1,474}$=56.9, P<0.001]. After only 1 day of fasting, there were significant differences between S and F population flies in the C and A groups (P<0.016, P<0.010, respectively; Fig. 2D). For all time points following 24 h of fasting, all S populations exhibited a reduction in PER compared with counterpart F population flies (P<0.01 for all groups). F flies reached the highest PER response at their maximal survival time, which occurred after ~6 days of fasting. The response of S flies was ~40–80% lower at the same time point. S flies also exhibited the highest response near their maximum survival time (18 days) at day 15 of fasting (Fig. 2D). Therefore, S flies are capable of eliciting PER responses equivalent to those of F flies, but S flies require a significantly longer fasting period than F flies to exhibit PER.

To measure feeding over a prolonged period, sucrose and yeast extract intake were measured in the capillary feeding (CAFÉ) assay (Fig. 2E; Ja et al., 2007; Masek and Keene, 2013). All flies were starved for 48 h prior to testing, and the total volume of either 5% sucrose or 5% yeast extract that was ingested over 6 h was measured. The consumption of yeast and sucrose was significantly higher in F population flies after only 10 and 30 min of feeding, respectively, compared with S flies, which reveals that there is reduced adult feeding in S flies (Fig. 2F; P<0.039 for yeast, P<0.013 for sucrose). These results confirm that flies selected for starvation resistance have reduced fasting-induced feeding in response to multiple dietary components. Three independent feeding assays that measured short-term feeding, reflexive feeding drive and prolonged food consumption confirm that starvation selection results in dramatically reduced feeding in fasted adults.

It has been previously shown that flies are capable of shifting their food preference in response to starvation (Stafford et al., 2012; Ribeiro and Dickson, 2010). When given a choice between equal

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concentrations of 5% yeast and 5% sucrose, flies prefer sucrose. A modified version of the CAFÉ assay was employed where flies were given a choice between 5% sucrose and 5% yeast extract (Fig. 3A). The consumption of sucrose relative to yeast extract was measured for 6 h. Both F and S flies preferred 5% sucrose over 5% yeast throughout the measurement (P<0.001; Fig. 3B). We sought to determine how fasting modifies preference between yeast and sucrose in flies selected for starvation resistance. We reduced the concentration of sucrose so that flies preferred yeast extract to sucrose. F and S population flies initially preferred yeast to sucrose at 50 mmol l^{-1} concentrations of sucrose, but gradually switched their preference to sugar over time. When fasted for an equal amount of time (3 days), F flies switched to sucrose preference between 30 and 60 min of feeding, whereas S flies had a preference shift that was delayed for 3 h or longer (significant difference in the slope; repeated measures ANCOVA: $F_{6,17}$=6.8, P=0.001; Fig. 3C). Quantification of sucrose–yeast preference indicates that both F and S flies display a shift between yeast and sugar preference, but S flies have a delayed preference shift. Therefore, we hypothesized that the slower shift in preference was due to enhanced starvation resistance in S flies. To test this hypothesis, we fasted S flies for an additional 3 days (total 6 days fasting) and the shift in yeast–sucrose preference occurred between 1 and 3 h (for the difference between 3 and 6 day fasted flies in S populations: Mann–Whitney U=25.0 and 17.0, P<0.05 and P<0.01 for 60 and 180 min, respectively, after Bonferroni correction; Fig. 3D). Therefore, the delayed yeast–sucrose preference shift is conserved in flies selected for starvation resistance and only occurs after prolonged starvation. These results reveal that a robust sucrose preference remains in S flies, suggesting that consumption, but not dietary preference, is altered in S flies.

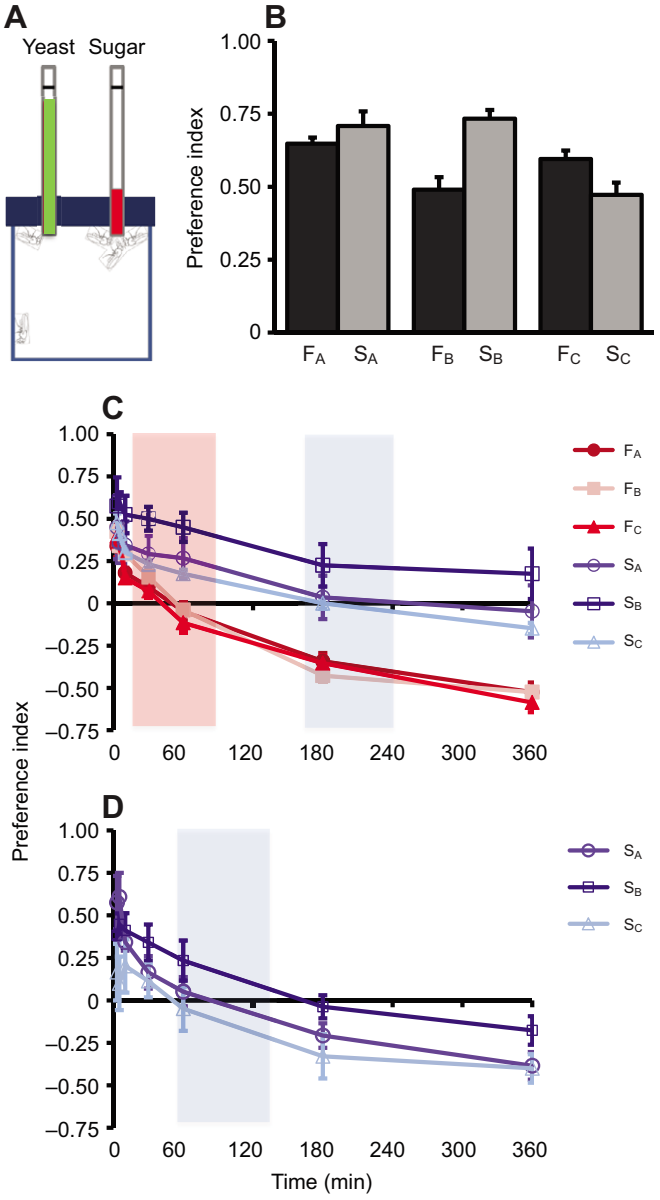

Fig. 3. Starvation selection does not change food choice. (A) Flies were provided with a choice between sucrose and yeast in the CAFÉ assay. (B) Quantification of total preference of 5% sucrose over 5% yeast extract reveals a strong sugar preference for F (black bars) and S flies (gray bars) (P<0.001 for all groups). (C) In a choice between 5% yeast and 50 mmol l^{-1} sucrose, 3 day fasted flies initially preferred yeast, but shifted their preference to sucrose over time. In F flies, the shift occurred between 30 and 60 min, whereas in S flies, the shift occurred after 3 h [slope difference between F and S lines, repeated measures ANCOVA (group×time interaction): $F_{6,17}$=6.8, P=0.001, N=12 for F and S populations]. (D) Fasting of S populations for 6 days resulted in a shift to sucrose preference between 1 and 3 h of feeding [between 3 and 6 days fasting in the S population (N=12 and 10, respectively): P<0.05 (Mann–Whitney U=25) and P<0.01 (U=17) at 60 and 180 min, respectively). Red and blue blocks in C and D denote the respective approximate switch from yeast to sucrose preference in F group and S group flies.

Sleep duration is enhanced in starvation-resistant flies

Sustained sleep results in energy conservation (Berger and Phillips, 1995; Zepelin et al., 1994). Therefore, a dramatic increase in the starvation resistance of S flies may, in part, be explained by prolonged sleep. We measured sleep and activity of both fed and starved S and F flies using the *Drosophila* Activity Monitoring (DAM) system (Fig. 4A). Sleep in flies can be inferred by determining inactivity bouts of ≥5 min (Shaw et al., 2000). Total sleep duration over 24 h on standard fly food was measured for S and F population flies. Both daytime and nighttime sleep was significantly increased in S flies ($F_{1,305}$=141.3, P<0.001), which raises the possibility that selection for starvation resistance favors long-sleeping flies (Fig. 4B). The total activity per waking minute did not differ between S and F population flies in the A group (*post hoc* test: t_{122}=–0.27, P=0.789), but was reduced in S_B and S_C groups compared with F controls, resulting in a significant change between F and S groups ($F_{1,305}$=30.1, P<0.001), suggesting that general lethargy may contribute to reduced sleep for some, but not all of the S populations (Fig. 4C).

The DAM system monitors reportedly generate less accurate sleep data compared with automated tracking (Zimmerman et al., 2008). To verify results obtained with infrared-based recordings, video recordings of flies were captured, and the activity of the flies was analyzed using automated tracking software (Fig. 4D). These experiments confirmed that sleep was significantly increased in S population flies compared with F controls ($F_{1,140}$=116.3, P<0.001, N=75 and 71 for control F and S populations, respectively; Fig. 4E). Analysis using tracking software indicated that this effect was not exclusively due to lethargy because waking activity did not differ in two of the three S groups compared with their respective F controls (*post hoc* test with Bonferroni correction for group A and B: t_{41}=0.1 and $t_{28.1}$=1.1, both P>0.05; group C: $t_{29.2}$=3.3, P<0.05; Fig. 4F). Taken together, these findings support the notion that increased sleep, and consequently, reduced energy expenditure contribute to the starvation resistance of S population flies, most probably through the conservation of energy stores.

Flies regulate sleep and activity in accordance with food availability and internal energy stores (Dus et al., 2011; Keene et al.,

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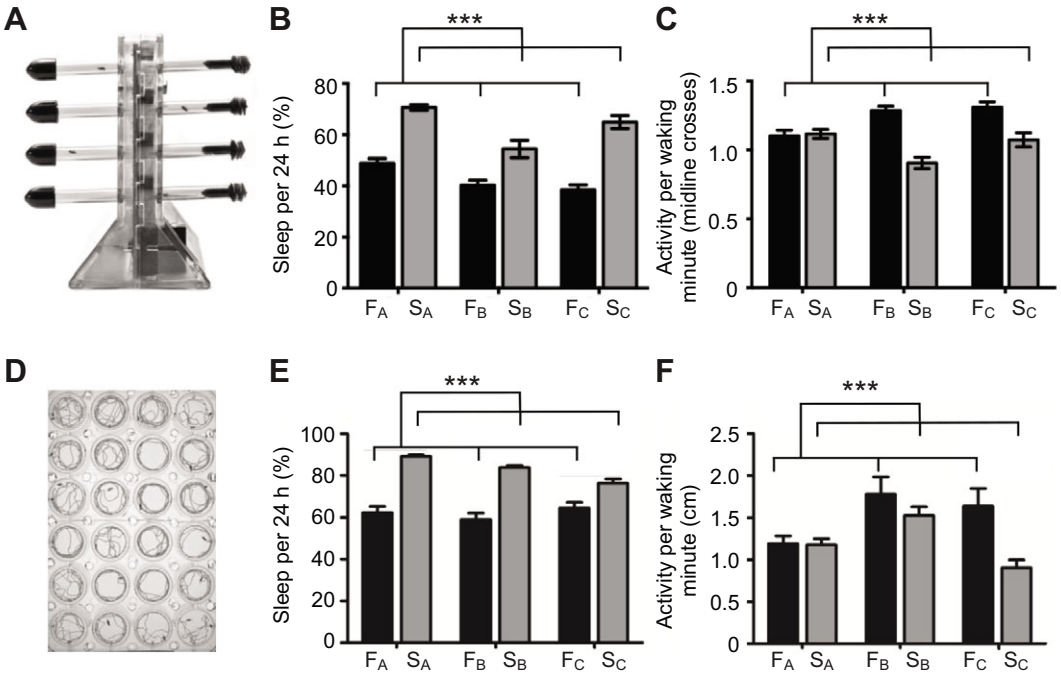

Fig. 4. Sleep is dramatically increased in flies selected for starvation resistance. (A) Activity was monitored by measuring the number of infrared-beam crossings in the DAM system. (B) Total sleep duration (percentage of 24 h) was measured for day 1 on food and subsequent days on agar. S flies slept more than F flies on food ($F_{1,305}$=141.3, P<0.001, N=190 and 121 for control F and S populations, respectively). (C) The A populations of S and F flies did not differ in their waking activity (t_{122}=-0.27, P=0.789), whereas S flies in the B and C groups were less active than F flies, and overall waking activity was reduced in S population flies ($F_{1,305}$=30.1, P<0.001, N=190 and 121 for F and S populations, respectively) when measured using the DAM system. (D) To more precisely quantify sleep, activity was recorded in a custom-built fly-tracking chamber. (E) Video tracking confirmed increased sleep duration in S group flies compared with F control flies ($F_{1,140}$=116.3, P<0.001 for all groups, N=75 and 71 for F and S populations). (F) Waking activity did not differ between the A and B groups of S flies compared with F flies when video analysis was used (*post hoc* test for group A and B: t_{41}=0.1 and $t_{28.1}$=1.1, respectively, both P>0.05); however, S flies as a whole were less active than F population flies ($F_{1,121}$=8.6, P=0.004, N=62 and 65 for F and S populations, respectively). ***P<0.001.

2010; Lee and Park, 2004). Highly inbred laboratory strains belonging to multiple *Drosophila* species acutely regulate sleep in response to starvation by suppressing sleep, presumably to forage for food (Keene et al., 2010; Thimgan et al., 2010). Therefore, starvation selection may elicit enhanced foraging behavior and reduced sleep. Alternatively, it is possible that starvation selection promotes behavioral quiescence because food is entirely absent. To distinguish these two possibilities, we measured sleep changes in response to starvation using the DAM system. Sleep was measured on food for day 1, after which flies were transferred to agar for fasting. Total sleep was then measured until <30% of the flies remained alive (Fig. 5A). We found that all three F control populations significantly suppressed sleep after only 24 h of fasting [repeated measures ANCOVA: $F_{3,234}$ (population×time)=33.0, P<0.001 between F and S populations], whereas starvation-induced sleep suppression was not observed in S population flies, indicating that selection for starvation resistance inhibits starvation-induced sleep suppression (linear regression: R^2=0.031, P>0.05 for all S populations). Starvation of S flies for a prolonged number of days (so that flies approached death by starvation) did not induce sleep suppression (Fig. 5A), indicating that the absence of sleep suppression was not due to a general shift in starvation-induced behavior.

Fly sleep is composed of individual sleep bouts, and starvation of inbred laboratory strains results in a reduced number of sleep bouts (McDonald and Keene, 2010). The average bout numbers in F and S populations were significantly different following 24 h of fasting [repeated measures ANCOVA (day×population): $F_{3,235}$=17.4, P<0.001; *post hoc* test between the first and second day (day×population): $F_{1,237}$=18.4, P<0.001], suggesting that the F population reduced bout number significantly more than the S population while fasting (Fig. 5B). Interestingly, the sleep bout number rebounded to baseline levels following five days of fasting in all three S populations, whereas in F populations, it remained reduced until death. These findings reveal that metabolic regulation of sleep is dramatically transformed in flies selected for starvation resistance. In addition to suppressing sleep, starved flies also increase their waking activity (Keene et al., 2010; Lee and Park, 2004). F population flies increased waking activity significantly more than S population flies during the first day of fasting [repeated measures ANCOVA (day×population): $F_{3,234}$=29.3, P<0.001; *post hoc* between the first and second day (day×population): $F_{1,236}$=85.1, P<0.001]. S_A flies did not increase waking activity for any of the days tested, and they reduced waking activity on a number of days following food deprivation (regression analysis for S_A: beta=–0.308, t=–3.7, P<0.001; Fig. 5C). Therefore, modulation of both sleep and total activity in response to fasting are dysregulated in flies selected for starvation resistance.

It is possible that the lack of starvation-induced sleep suppression is due to an inability to generally modulate sleep in response to stress. Alternatively, the lack of sleep suppression in the S population flies could be specific to starvation. To distinguish these two possibilities, we tested sleep-deprived flies and measured daytime sleep rebound. After 12 h of manual sleep deprivation (shaking flies every 3–4 min) results in near complete loss of nighttime sleep and induces a dramatic rebound the following day (Fig. 5D). All three S and F populations displayed a significant sleep rebound compared with non-deprived groups (P<0.001) (Fig. 5D;

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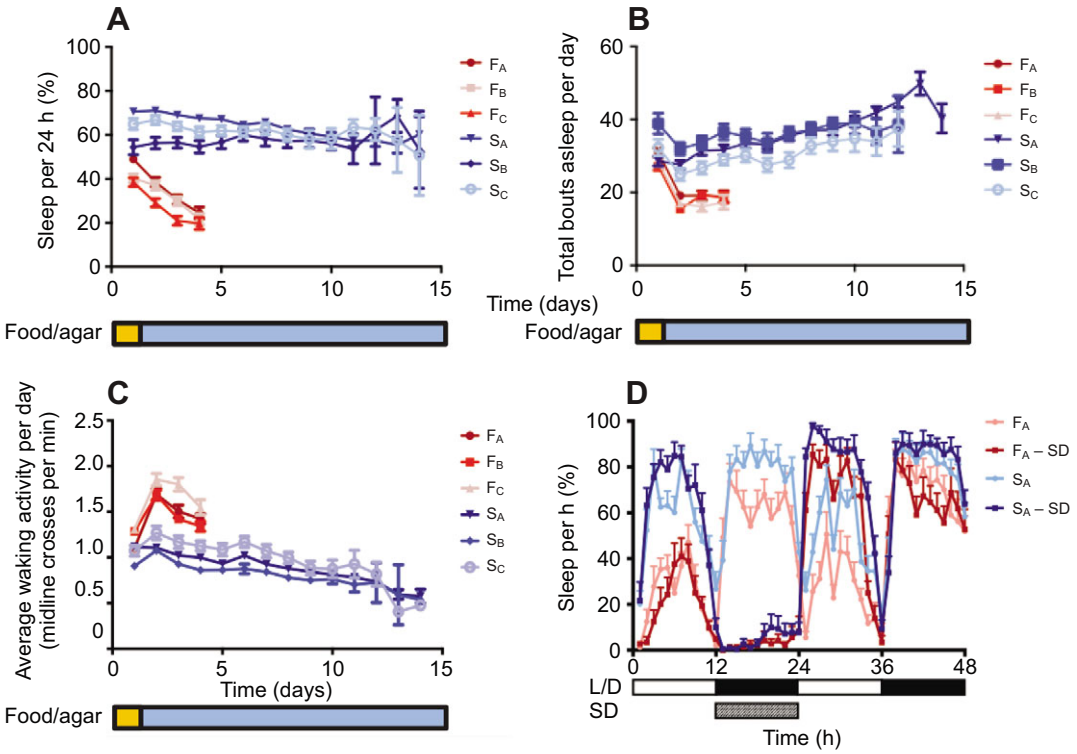

Fig. 5. Starvation-resistant flies do not suppress sleep in response to starvation. (A) Sleep was measured in flies housed on agar alone (white bar). F flies suppress sleep after fasting [$F_{3,234}$ (population×time)=33.0, P<0.001, N=119 for F and S populations], whereas no difference in sleep was observed in S flies over 14 days (R^2=0.031, P>0.05). (B) Starvation resulted in reduced bout number following 24 h of fasting in F flies [ANCOVA (day×population): $F_{3,235}$=17.4, P<0.001, N=120 and 119 for F and S populations, respectively; *post hoc* test between the first and second day (day×population): $F_{1,237}$=18.4, P<0.001]. (C) F flies increase waking activity following 1 day of fasting upon comparison with S flies [ANCOVA (day×population): $F_{3,234}$=29.3, P<0.001, N=119 for F and S populations]. (D) F and S flies were sleep-deprived (SD) from hours 12–24 (hashed bar) during the dark period. Both F and S flies display much more robust sleep rebounds compared to undisturbed controls (P<0.001 for all groups). L/D, light (white) and dark (black).

supplementary material Fig. S1). This indicates that the prolonged sleep of flies selected for starvation resistance is due to elevated sleep need and that delayed starvation-induced sleep suppression is not due to a general inability to modulate sleep. These results support the notion that selection for starvation resistance promotes an adaptive increase in sleep need and delayed starvation-induced sleep suppression. These findings suggest that increased sleep results in energy conservation when food is completely absent, thereby contributing to starvation resistance.

Enhanced sleep duration is not linked to energy stores or body size

Both feeding and sleep in *Drosophila* are regulated by metabolic state and the availability of energy stores (Keene et al., 2010; Thimgan et al., 2010). It is possible that the molecular basis for increased sleep duration in S flies is related to either elevated energy stores or modified genetic background that is independent of energy stores. To distinguish these possibilities, we rescued the obese phenotype of S population flies by shortening their larval feeding time. The larvae of S population groups were taken off of food and placed onto agar at the time when third-instar larvae from the F groups stopped feeding and wandered towards their pupation site (Fig. 6A). This manipulation triggers premature pupation of S larvae and results in S larvae with energy stores comparable to those of F flies (C. M. Hardy and A.G.G., personal communication). These flies were then transferred to food immediately post-eclosion and tested for sleep or feeding at 3 to 5 days of age. Reduction of the obese phenotype in S flies did not restore fasting-induced feeding ($F_{1,99}$=0.01, P=0.910, N=54 and 51 for S and rescued S populations, respectively; Fig. 6B) or restore normal sleep duration when compared with non-reduced controls ($F_{1,57}$=5.0, P=0.029, N=32 and 31 for S and rescued S populations, respectively; Fig. 6C). Control F flies that were transferred to agar did not increase their feeding response to starvation or exhibit reduced changes in sleep, indicating that the lack of rescue observed in feeding and sleep assays was not due to confounding effects of transferring flies to agar (data not shown). Our results suggest that the reduced feeding and increased sleep are due to genetic changes induced by selection for starvation resistance. These changes are independent from elevated lipid levels in S population flies.

Distinct genetic factor(s) regulate sleep and feeding changes in starvation-resistant flies

We next sought to determine whether shared genetic architecture regulates the reduced feeding and increased sleep behaviors that result from starvation selection. To address this question, we generated F2 S-F hybrid flies by mating parental lines from each of the three group pairs. Sleep was then recorded for 24 h in the DAM system, followed by starvation and food-consumption analysis in individual flies using the blue-dye feeding assay (Fig. 7A). Both sleep on food and food consumption were correlated for individual flies. For all three groups tested, there was no correlation between sleep and feeding (S_A-F_A hybrid: R^2=0.032; S_B-F_B hybrid: R^2=0.013; S_C-F_C hybrid: R^2=0.028; Fig. 7B), suggesting that distinct genetic architectures regulate the increased sleep and reduced feeding behaviors that are present in S flies. These findings reveal that in

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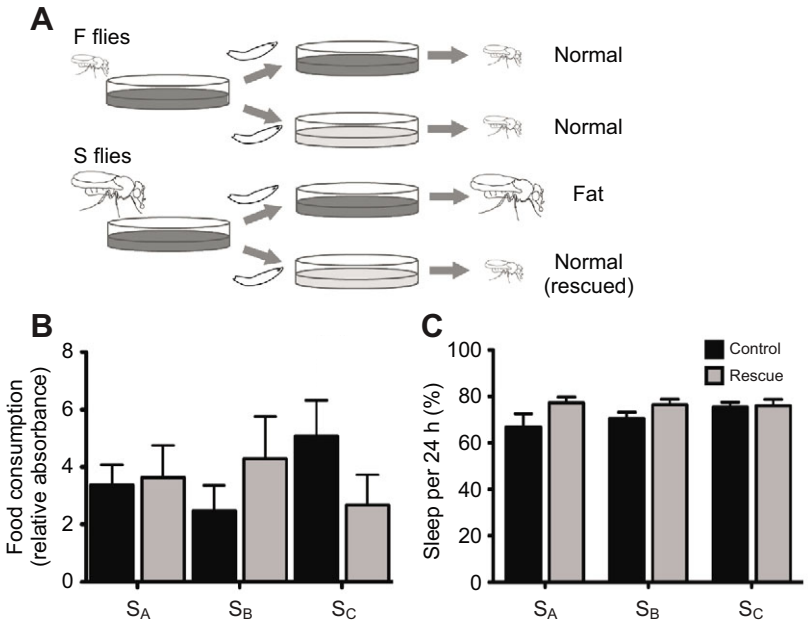

Fig. 6. Feeding and sleep phenotypes in S flies are not due to elevated energy stores. (A) Early third-instar larvae from S populations were transferred to agar at the same time that F larvae leave food as wandering third-instar larvae. Control S larvae were maintained on food throughout development. (B) Single meal consumption did not differ between rescued and control S flies ($F_{1,99}$=0.01, P=0.910, N=54 and 51 for S and rescued S populations, respectively). (C) Total sleep duration over 24 h was restored in rescued S flies ($F_{1,57}$=5.0, P=0.029, N=32 and 31 for S and rescued S population, respectively).

addition to selecting for changes in lipid content and metabolic function, starvation selection independently transforms the genetic architecture that controls both sleep and feeding in *Drosophila*.

DISCUSSION

Across phyla, both sleep and feeding strategies are tightly linked to an animal's food availability and evolutionary history. Most studies examining the evolutionary basis for sleep and feeding have relied on cross-species variation, but directly addressing the evolutionary relationship between these traits has been difficult in a laboratory setting (Allada and Siegel, 2008). To address this issue, we have utilized experimental selection to examine the evolutionary effects of limited food availability on sleep and feeding. Selecting for starvation resistance over 60 generations resulted in flies with a larger body size, increased lipid content and decreased metabolic rates (Fig. 1; L.A.R. and A.G.G., submitted). It has been proposed that flies can also develop starvation resistance by reducing energy expenditure (Rion and Kawecki, 2007). Our findings directly demonstrate that flies increase sleep and reduce feeding behaviors to limit energy expenditure. Consequently, the behavioral changes in sleep and foraging are likely to represent adaptation to decrease energy expenditure, thereby further increasing starvation resistance.

Prolonged sleep duration and a reduction in total activity are likely to promote energy conservation (Siegel, 2008). We employed both infrared tracking with DAM system monitors and video analysis to measure total sleep in flies that had been selected for starvation resistance. In both assays the three S populations tested slept significantly more than F population flies, supporting the notion that prolonged sleep contributes to starvation resistance (Fig. 4B,E). The activity per waking minute was variable between

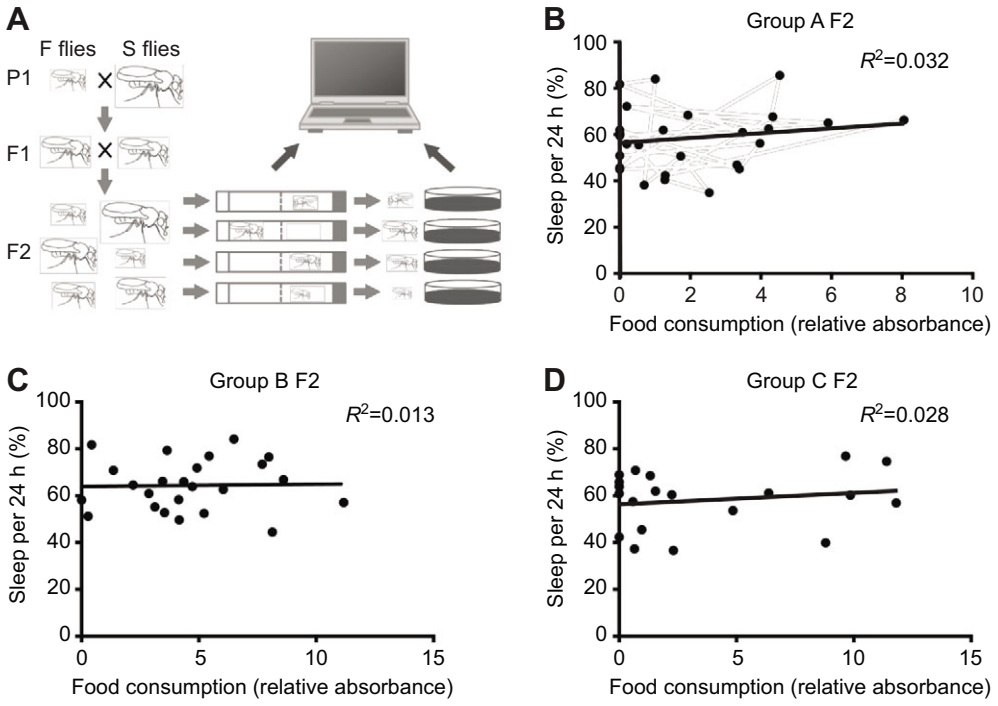

Fig. 7. Distinct genetic factors regulate selection-driven alterations in feeding and sleep. (A) F2 progeny were generated by crossing F and S flies. Individuals were then tested for sleep on food, followed by feeding. (B–D) Correlation analysis for F2 hybrids from three distinct sets of individually derived strains display a lack of correlation between 24 h sleep duration and food consumption (S_A-F_A hybrid: R^2=0.032; S_B-F_B hybrid: R^2=0.013; S_C-F_C hybrid: R^2=0.028).

selection groups with S_A group flies showing similar activity to F_A group flies and S_C group flies showing reduced waking activity (Fig. 4C,F). These findings raise the possibility that there are multiple mechanisms for energy conservation where flies reduce activity while awake and increase total sleep duration. The elevated sleep of S group flies may be due to an increase in sleep need because 12 h of sleep deprivation resulted in a dramatic homeostatic sleep rebound in all three groups of S flies. However, it is difficult to directly compare sleep rebound in F and S population flies because sleep duration in undisturbed flies differs dramatically (Fig. 5D; supplementary material Fig. S1). It is also possible that arousal is constitutively lower in S group flies, resulting in enhanced sleep in S group flies and a robust response to sleep deprivation. Therefore, changes in sleep and locomotory behavior appear to contribute to starvation resistance.

Sleep and feeding represent mutually exclusive behaviors, and evidence suggests there are trade-offs between these processes. In mammals, sleep loss increases feeding, whereas starved flies and mammals suppress sleep, presumably to search for food (Horne, 2009; McDonald and Keene, 2010). We found that S flies fail to suppress sleep, even after 14 days of fasting, raising the possibility that S group flies have lost the ability to modify behavior in response to fasting. However, PER was increased in S group flies that had been starved for 10 days, indicating that feeding, but not sleep, can be modulated by starvation (Fig. 2D). Feeding and locomotion both represent foraging-related behaviors, and previous findings indicate that there is a trade-off between foraging and starvation resistance. Flies that are mutant for activity-regulated cytoskeleton-associated protein (ARC1) do not exhibit a hyperlocomotor response to fasting and show increased starvation resistance, whereas mutation of the circadian genes *Clock* and *cycle* exacerbates starvation-induced sleep suppression and impairs starvation resistance (Keene et al., 2010; Mattaliano et al., 2007). Therefore, flies selected for starvation resistance provide an excellent model for understanding how sleep and metabolism are interconnected, because they have become uncoupled in the S flies.

Starvation resistance is positively correlated with lipid content across different *Drosophila* species, suggesting that triglyceride stores are a key regulator of survival in response to nutrient scarcity (Ballard et al., 2008; Sharmila Bharathi et al., 2003; Van Herrewege and David, 1997). Further, triglyceride levels and the fat body are known regulators of both sleep and feeding behaviors; however, little is known regarding the role of lipid levels in regulating the behavioral changes associated with flies that have been selected for starvation resistance (Thimgan et al., 2010; Subramanian et al., 2013; Xu et al., 2008). We reduced the obesity phenotype in S population flies by removing third-instar S larvae from food prematurely to eliminate the prolonged development and lipid accumulation present in these flies. Reducing the elevated lipid levels and body size of S flies did not restore normal sleep or feeding, suggesting that enhanced sleep and reduced feeding of S population flies was not directly due to elevated triglyceride levels. Therefore, it appears the energy conserving changes in sleep are not the direct result of elevated fat stores. These findings do not rule out the role of fat bodies in behavioral changes observed in S flies. It is possible that genetic changes result in modified regulation of fat-body-secreted proteins, thereby modulating sleep independently of triglyceride levels. Short-sleeping selected flies have a reduced lifespan and elevated levels of the sleep-suppressing neurotransmitter dopamine (Seugnet et al., 2009). Interestingly, both the S flies described in this study and the short-sleeping selected flies have elevated lipid levels. This raises the possibility that elevated lipid levels are a general attribute to selection triggered by selection under stressful conditions, rather than a specific regulator of sleep.

We tested individual F2 S-F hybrid flies for both sleep and feeding and found no correlation between these behaviors, suggesting further that distinct genetic factor(s) regulate the increased sleep and reduced feeding present in S flies. Although the genetic basis for increased feeding and reduced sleep are not known, previous studies have identified many single-gene mutations that are required for sleep or feeding. For example, the neuropeptide Y ortholog Neuropeptide F and *hugin* promote feeding (Melcher and Pankratz, 2005; Wu et al., 2003). A number of sleep-promoting genes have also been identified, including *shaker*, *sleepless* and *Cyclin A* (Cirelli et al., 2005; Koh et al., 2008; Rogulja and Young, 2012). It is possible that differential expression of these genes plays a role in the sleep and feeding changes observed in S population flies. It will be of particular interest to determine if any of the genes identified in that study are dysregulated in S population flies.

In addition to experimental evolution, a number of studies have harnessed the power of natural variation to determine the evolutionary mechanisms underlying starvation resistance. Geographic location appears to potently regulate starvation resistance and metabolic rate in outbred *Drosophila*. Populations of *D. melanogaster* in northern latitudes of North America are more starvation resistant than southern populations, raising the possibility that variation in food availability or overwintering phenotypes underlies naturally occurring differences in starvation response (Schmidt and Paaby, 2008; Schmidt et al., 2005). Although the effect of geographic location on sleep and feeding has not been systematically studied, it will be interesting to look at sleep and feeding in geographically independent populations.

Genomic approaches may complement studies using inbred fly lines in the search for the genetic basis underlying the sleep and feeding changes observed in S population flies. Highly inbred lines of wild-caught *Drosophila* have also been used to identify the genetic basis of sleep and starvation resistance. The *Drosophila* Genome Resource Project (DGRP) contains fully sequenced genomes and full body RNA-sequencing analysis that can be used for genome-wide association studies to map the genetic basis for behavioral traits (Mackay et al., 2012). This approach has been employed to identify loci linked to energy stores, sleep and starvation resistance (Harbison et al., 2004; Harbison et al., 2009; Jumbo-Lucioni et al., 2010). Analysis of quantitative trait transcripts for sleep duration identified *Akt1*, which regulates metabolic function and insulin–TOR signaling (Harbison et al., 2009; Kockel et al., 2010). The cAMP phosphodiesterase *rutabaga* has been implicated in sleep, and the expression level through quantitative trait transcript analysis is linked to triacylglyceride levels (Hendricks et al., 2001; Jumbo-Lucioni et al., 2010). Testing S-F F2 hybrids revealed a lack of correlation between sleep and feeding, suggesting that these traits are regulated by distinct genetic architecture. Future work examining the expression levels of transcripts identified in DGRP studies for transcripts that are functionally associated with sleep and metabolism may provide candidate regulators of sleep and feeding changes in starvation resistant flies.

Selection studies have typically lacked the power to identify individual genes that regulate behavior. However, a number of genomic approaches have identified specific biological processes through experimental evolution. A recent study performed whole-genome sequencing on lines of *Drosophila* that had been selected for accelerated development (Burke et al., 2010). Performing a similar analysis of the S lines described in this study may help to

identify genes involved in both sleep and feeding. Our results suggest that experimental selection for starvation resistance modifies adult sleep and feeding behaviors. Increased sleep probably contributes to energy conservation and the lower metabolic rate of S flies. Decreased feeding and foraging behaviors may be the result of selection in conditions devoid of food. The uncoupling of these potentially related behaviors entails that these behavioral modifications in S population flies occur through independent mechanisms. Investigating the molecular mechanisms through which sleep and feeding are modified in S flies may be used to discover new genes that regulate sleep and feeding in both flies and mammals.

Taken together, these findings reveal dramatic behavioral changes associated with selection for starvation resistance. These experiments highlight the role of behavioral changes, in addition to metabolic and developmental changes, for conferring starvation resistance. Further, the dramatic changes in feeding, sleep and metabolic regulation of behavior that are present in S group flies provide a model system for understanding the genetic basis for these behaviors

MATERIALS AND METHODS

Generation of starvation-resistant flies

The wild-derived *D. melanogaster* stocks used in this study were collected from Terhune Orchards, Princeton, NJ, USA, in 1999 and were maintained as outbred stocks at 25°C on standard corn meal medium since this time. The S and F populations were derived from two control treatments for desiccation-selected populations described previously by Gefen et al. (Gefen et al., 2006). Desiccation selection was performed by depriving replicated populations of access to food and water. Starved control treatment comprised access to water but not food for the same length of time as the desiccation-selected populations, resulting in <20% mortality in the controls. A second set of control populations was provided *ad libitum* food and water. The starved control populations were used to found populations subjected to severe starvation selection (S populations). For the selection process, approximately 8000 experimental flies for each of the three starvation-selected groups were maintained in constant light at room temperature (~23°C) on 1% agar until only 15–20% of the original population survived. Surviving flies were then placed on food to lay eggs. The next generation of adults was selected for starvation resistance in the same manner. Flies assayed for the behavior experiments described in this manuscript ranged between generation 55 and 70 of selection. F populations were derived from the fed control populations in the desiccation selection experiment (Gefen et al., 2006). They were maintained on food, whereas the S populations were starved. There were three replicate S populations (designated S_A, S_B and S_C) and three fed control populations (F_A, F_B and F_C). All selection occurred in the laboratory of A.G.

Drosophila maintenance

Flies taken off of the selection process for behavioral experiments were maintained and tested in humidified incubators at 25°C and under 65% humidity (Powers Scientific). Flies were reared on a 12 h:12 h light–dark cycle for two to six generations following selection prior to behavioral analysis. All flies were maintained on Jazz-Mix *Drosophila* food (Fisher Scientific).

Sleep and activity analysis

Activity monitoring using *Drosophila* Activity Monitoring system

Fly activity was monitored using DAM2 *Drosophila* activity monitors (Trikinetics, Waltham, MA, USA) as previously described (Hendricks et al., 2000; Shaw et al., 2000). Flies were briefly anesthetized using cold-shock within 1 h of lights on at Zeitgeber time 0 (ZT0) and placed into plastic tubes containing standard food. The DAM system monitors activity by detecting infrared beam crossings for each animal. These data were used to calculate sleep information by extracting immobility bouts of 5 min using the *Drosophila* sleep counting macro (Pfeiffenberger et al., 2010). Multiple variables of sleep were analyzed, including total sleep duration, sleep bout number and average sleep bout length as previously described (Pfeiffenberger et al., 2010; Pitman et al., 2006). For experiments examining the effects of starvation on sleep, activity was recorded for 1 day on food, prior to transferring flies into tubes containing 1% agar (Fisher Scientific). Flies were then transferred every 7 days onto fresh agar tubes for the remainder of the experiment until <30% of the flies survived. For sleep deprivation experiments, flies were shaken in DAM2 monitors every 3–4 min for 12 h from ZT12 (onset of darkness) through ZT0 (onset of light). Stimulus was applied using a vortexer (Fisher Scientific, MultiTube Vortexer) with a custom-milled plate to hold DAM2 monitors and a repeat cycle relay switch (Macromatic, TR63122). Sleep rebound was measured the following day from ZT0 to ZT12.

Video-tracking analysis

Fly activity was recorded using a custom-built video acquisition system similar to a previously published system in fish (Duboué et al., 2011). Flies were anesthetized using cold-shock and loaded into standard 24-well tissue culture plates (BD Biosciences, 351147), with each well containing either 5% sucrose dissolved in 1% agar (fed group) or 1% agar alone (starved group). The plates were placed in a chamber illuminated with white (6500K) LED lights (Environmental Lights Inc., San Diego, CA, USA, product no. dlrf3528-120-8-kit) on a 12 h:12 h light–dark cycle that had constant illumination from 850–880 nm infra-red (IR) lights (Environmental Lights Inc., product no. irrf850-390). Video was recorded using an ICD-49 camera (Ikegami Tsushinki Co., Japan) fitted with an IR-transmitting lens (Computar Inc., Los Angeles, CA, USA, Vari Focal H3Z4512 CS-IR 4.5-12.5 mm F 1.2 TV lens). An IR high-pass filter (Edmund Optics Worldwide, Barrington, NJ, USA, filter optcast IR 5×7 in, part no. 46,620) was placed between the camera and the lens to block visible light. Video was recorded at a resolution of 525 lines at 59.94 Hz, 2:1 interlace. Fly activity was analyzed using Ethovision XT 9.0 video-tracking software (Noldus Inc., Leesburg, VA, USA). Sleep was calculated by measuring bouts of inactivity ≥5 min using a previously described Microsoft Excel macro (Duboué et al., 2011)

Survival index

Flies were starved on 1% agar in individual tubes, and their activity was recorded in DAM2 monitors. Activity was measured using the sleep counting macro (Pfeiffenberger et al., 2010). Death was manually determined as the last activity time point from the final recorded activity bout for each individual fly. For analysis, we applied Kaplan–Meier analysis by grouping each control and starvation resistant population. N=32, 32, 32, 31, 16, 15 flies from F_A, F_B, F_C, S_A, S_B and S_C populations, respectively.

Feeding assays

Proboscis extension reflex

Flies at 3 to 5 days old were collected and placed on fresh food for 24 h, then starved for the designated period of time in vials containing wet Kimwipe paper (Kimberly-Clark Corporation, Irving, TX, USA). Flies were then anesthetized under CO_2, glued with nail polish (catalog no. 72180, Electron Microscopy Science) on a microscopy slide to their thorax and wings, leaving heads and legs unconstrained (Keene and Masek, 2012). Following 3–6 h recovery in a humidified chamber, the slide was mounted vertically under the dissecting microscope (Leica, S6E, Wetzlar, Germany) and PER was observed. PER induction was performed as described previously (Keene and Masek, 2012; Masek and Scott, 2010). Briefly, flies were satiated with water before and during experiments. Flies that did not water satiate within 5 min were excluded from the experiment. A 1 ml syringe (Tuberculin, FD&C Blue no. 1, Spectrum Laboratory Chemicals, New Brunswick, NJ, USA) with an attached pipette tip (TipOne, no. 1111-0200, USA Scientific, Ocala, FL, USA) was used for tastant presentation. Tastant was manually applied to tarsi for 2 to 3 seconds three times with 10 second inter-trial intervals, and the number of full proboscis extensions was recorded (Chabaud et al., 2006; Wang et al., 2004). Tarsi were then washed with distilled water between applications of different tastants and flies were allowed to drink water during the experiment *ad libitum*. Each fly was

assayed for response to multiple tastants. PER response was calculated as a percentage of proboscis extensions to the total number of tastant stimulations to tarsi (Keene and Masek, 2012).

Two-choice capillary feeding (CAFÉ) assay

A modified volumetric drinking assay was used to test food preference (Ja et al., 2007; Masek and Keene, 2013). Flies were allowed to drink two solutions that were presented in capillaries (WPI, Sarasota, FL, USA, no. 1B150F-4 ID 1 mm, OD 1.5 mm, with filament) attached to an empty food vial, and vials were placed at a 45 deg angle. The openings of the capillaries were aligned with the ceiling of the vial. Following a defined fasting period, 30–60 flies were placed into a vial, and food consumption was measured. The volume consumed was calculated as the length of liquid missing from the capillary multiplied by the cross-section of the inner diameter of the capillary. All measurements were correlated for missing liquid due to evaporation using control capillary tubes without flies. Consumption was measured every hour following the introduction of flies into the assay. Taste compounds were mixed with Allura Red food dye (Federal Food, Drug, and Cosmetic Act, red no. 40, Sigma-Aldrich, St Louis, MO, USA) to a concentration of 3 µl ml^{-1} for better visibility in the capillary tube. Following the conclusion of the assay, flies were anesthetized and the number of flies in each vial was counted. Total consumption per fly was measured as volume consumed in each capillary divided by the number of live flies in the vial. The preference index was calculated as volume consumed from one capillary minus the volume consumed from the second capillary with control solution, divided by the total volume consumed.

Blue-dye feeding assay

Short-term food intake was measured as previously described (Wong et al., 2009). Briefly, flies were starved for 24 or 48 h on wet Kimwipes or maintained on standard fly food. At ZT0, flies were transferred to food vials containing 1% agar, 5% sucrose and 2.5% blue dye Federal Food, Drug, and Cosmetic Act, blue dye no. 1). Following 30 min of feeding, flies were flash frozen on dry ice and individually homogenized in 400 µL PBS (pH 7.4, Ambion). Color spectrophotometry was then used to measure absorbance at 655 nm in a 96-well plate reader (Millipore, iMark, Billerica, MA, USA). Baseline absorbance was determined by subtracting the absorbance measured in non-dye-fed flies from each experimental sample.

Standardizing larval feeding time between S and F flies

Standardizing larval feeding time in S and F flies (larval feeding and development is longer in S flies) was accomplished by removing S third-instar larvae from food prior to the wandering third-instar phase, at the time F larvae were wandering and pupating. Larvae were raised on standard cornmeal containing 0.05% bromophenol blue (Sigma-Aldrich) for larval staging (Andres and Thummel, 1994). Third-instar F larvae were identified by the presence of a clear gut, indicating the larvae were close to entering the wandering third-instar phase. Age matched third-instar S larvae with branched spiracles were then removed from food and placed onto 1% agar to shorten larval feeding time to that observed in F flies. As a handling control, F larvae were also moved and placed onto fresh food. Adult S flies from the experimentally shortened larval feeding group were then transferred from agar to standard fly food immediately following eclosion. These flies were then maintained on standard food until behavioral experiments were started.

Statistics

Statistical analyses were performed using InStat software (GraphPad Software 5.0 Inc.) or IBM SPSS 22.0 software (IBM, Somers, NY, USA). We employed two-way ANOVA for the most of comparative analysis (Fig. 2B, Fig. 3B, Fig. 4, Fig. 6) followed by *post hoc* analysis if it was necessary. In the slope analysis, we used ANCOVA to compare the slopes of grouped F (F_A, F_B and F_C) and S (S_A, S_B and S_C) populations. The statistics scores for the interaction (population×time) were reported for significant difference between slopes. For PER experiments, most tested groups violated the assumption of the normal distribution. Therefore, all data were analyzed with nonparametric statistics. All experiments include data from >18 flies. For PER experiments, each fly was sampled three times with the same stimulus. The response was binary (PER yes or no), and these three responses were pooled for values ranging from 0 to 3. The Kruskal–Wallis test (nonparametric ANOVA) was performed on the raw data from single flies and Dunn's multiple comparisons test was used to compare different groups. For the capillary feeding assay, 30–60 flies were used per tube and 4–20 tubes per group were tested. The Wilcoxon signed rank test (nonparametric) with two-tailed *P*-value was used to test significance on single groups. For comparing the switch between yeast and sucrose preference; linear regression was used to determine the time point of the switch and to compare the slopes of F and S populations. In figures, graph bars are mean values and error bars represent s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

P.M. and A.M. performed all feeding-related experiments. K.M., W.B. and C.M. performed sleep and starvation resistance experiments. M.Y. aided with experimental design and statistical analysis. L.R. generated starvation-resistant flies. A.G. and A.C.K. aided in experimental design and analysis of all experiments. The manuscript was drafted by P.M., L.R., W.B., A.G. and A.C.K.

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Supplementary material

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