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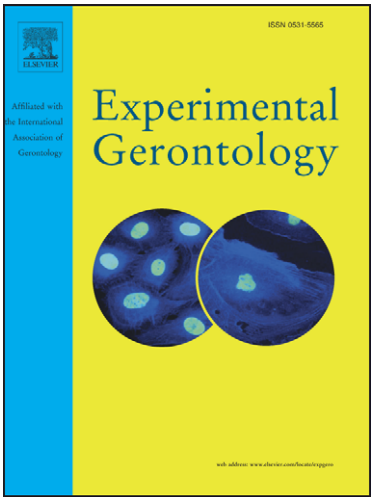

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Does Dietary Restriction Reduce Life Span in Male Fruit-feeding Butterflies?

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Abstract: Male life history and resource allocation is not frequently studied in aging and life span research. Here we verify that males of long-lived fruit-feeding butterfly species have reduced longevity on restricted diets (Beck 2007 Oecologia), in contrast to the common finding of longevity extension in dietary restriction experiments in *Drosophila* and some other organisms. Males of some of the most long-lived species of fruit-feeding butterflies were collected from Kibale Forest, Uganda, and kept on diets of either sugar or mashed banana. Seven out of eight species had non-significantly longer life spans on mashed banana diets. Data analysis using a time-varying Cox-model with species as covariate showed that males had reduced survival on the sugar diet during the first 35 days of captive life, but the effect was absent or reversed at more advanced ages. These results challenge the generality of dietary restriction as a way to extend life span in animals. We argue that such studies on males are promising tools for better understanding life history evolution and aging because males display a wider variety of tactics for obtaining reproductive success than females.

Key-words: mating tactics, reproduction, time-varying Cox-model, wing-wear

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Introduction

The majority of studies on effects of dietary restriction on longevity show life span extension under dietary restriction (Partridge et al., 2005), including model organisms ranging from yeasts (e.g., *Saccharomyces cerevisaie* Lin et al., 2002) to invertebrates (e. g., the nematode *Caenorhabditis elegans*Walker et al., 2005) and mammals (e.g., the rhesus monkey, *Macaca mulatta* Lane et al., 2004). Common belief that similar mechanisms can be inherent to humans stimulates intense scrutiny of the longevity benefits in model organisms (see recent review in Kennedy et al., 2007). However, recent studies that better represent the integrative nature of diet and resource allocation (using dietary restriction gradients, analyses of body composition, or measurement or control of food intake) show that these manipulations merely scratch the surface of the biological changes that are induced by diet (Carey et al., 2008a; Lee et al., 2008; Maklakov et al., 2008; Skorupa et al., 2008). Important model organisms in this field, such as lab rats, *Drosophila melanogaster* and Tephritid flies, have an evolutionary history on more or less complete diets.

The evolutionary history of Lepidoptera is probably dominated by adult diets consisting mainly of nectar sugars (Barth, 1991). Therefore, sugar solutions are regarded as the control to test for the effects of added amino acids or other nutrients on reproduction in this order. Usually, these additions do not affect the life span of female butterflies (Fischer and Fiedler, 2001; Hill and Pierce, 1989; Mevi-Schütz and Erhardt, 2003, 2005; Romeis and Wackers, 2002). A remarkable exception is found in the pollen feeding *Heliconius* butterflies where females deprived of pollen (which supplies them with amino acids for egg-production, O'Brien et al., 2003) live much shorter (Dunlap-

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Pianka, 1979; Dunlap-Pianka et al., 1977; Gilbert, 1972). Thus, while *Heliconius* diet is more complete like in *Drosophila*, dietary restriction via changes in diet composition had the opposite effect from that seen in *Drosophila*.

Recent data suggest that the life histories of fruit-feeding butterflies may tend towards reliance on sources of amino acids in the adult diet (Molleman et al., 2009), and Beck and Fiedler (2009) summarized circumstantial evidence for amino acids in the adult diet playing a crucial role in extended longevity in butterflies. Within this feeding guild, small effects of amino acids on longevity were found in *B. anynana* where females being additionally fed amino acids had lower survival rates compared to females fed on sugar only or banana (Bauerfeind and Fischer, 2005; Molleman et al., 2008b). In the larger *Charaxes fulvescens*, females fed banana lived shorter than those feeding on a sugar solution, while addition of amino acids to the sugar solution did not significantly affect longevity (Molleman et al., 2008a). Diet significantly affected mortality in captivity in a time-dependent manner in female *Euphaedra* butterflies, where butterflies fed a sugar-only diet initially survived better than those fed banana (see Table 1 and discussion for more data and details; Molleman et al., 2009).

Studies on life span effects of caloric restriction in Lepidoptera are scarce. In the butterfly *Speyeria mormonia,* adult survival was lower in both males and females subjected to larval semi-starvation (Boggs and Freeman, 2005), while adult caloric restriction had no effect on male or female life span but reduced female fecundity (Boggs and Ross, 1993) and male rates of spermatophore formation (Boggs unpub.). Individuals of *Jalmenus evagoras* attained the greatest longevity on a medium (25% wt/wt) sugar diet

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(Hill and Pierce, 1989). In the fruit-feeding butterfly *Bicyclus anynana*, life span is reduced under caloric restriction (Ferkau and Fischer, 2006; Molleman et al., 2008b).

Because information on life history of females is putatively most important for demography and population biology, and insect female reproduction is more readily measured in the laboratory (number of eggs), male life histories and resource allocation strategies are less frequently studied. Males, on the other hand, have attracted much attention from ethologists because of the wide range of mating tactics they can adopt (i.e. Alcock, 1994; Arlet et al., 2008; Waltz and Wolf, 1984). Therefore, the study of male life histories offers largely ignored opportunities to learn more about the evolution of life histories and the interrelatedness of behavior and life history.

Beck (2007) compared survival in captivity on a sugar solution versus an amino acid enriched sugar solution (butterflies also had access to salt) on Bornean wild-caught rainforest butterflies and caught mainly males. He found that males of some long lived butterfly species (including the fruit-feeding *Faunis gracilis* Morphinae) lived significantly longer when they had access to amino acids. His sample sizes per species and diet treatment varied between 5 and 19, while life spans ranged from 2 days to 4 weeks, and data were analyzed using two-tailed *t*-tests on log-transformed life span data.

For the one fruit-feeding butterfly species included (which responded significantly to amino acids), there were only 5 individuals in each diet treatment (Beck, 2007). Therefore, we further test the hypothesis that long-lived fruit-feeding butterfly males live longer when they have access to a diet that contains amino acids, using larger sample sizes and more appropriate statistical tools.

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Experimental methods

We used the two *Euphaedra* species with the longest life span records (Molleman et al., 2007) and several large *Charaxes* species (Table 1: Molleman et al., 2005a). Male butterflies were collected from Kibale National Park in April-May 2007 using baited traps, regardless of wing-wear, and kept singly in cages at Makerere University Biological Field Station. The locally manufactured cages were cylindrical (30 cm height, 21 cm width), made of fine mesh and 2 metal rings and fitted with a zipper. Butterflies and cages were numbered and hung in a room with four mesh covered windows (allowing ventilation and indirect sunlight) and a high (3-4 meters) wooden ceiling under a tin roof.

Wing length was measured using a ruler, and percent scale loss and wing surface loss were estimated and any missing appendages were recorded. Individuals were randomly assigned to either a mashed banana or a 10% (wt:vol) sugar solution diet. The mashed banana was prepared by slicing and mashing about 30 bananas in a clean bucket. Bananas contain about 1 gram of protein and 14 grams of sugar per 100 grams (NutritionData, 2008), and their sugar composition is very variable with about 50% sucrose, 25% glucose and 25% fructose (Molleman et al., 2008b). The sugar solution was made of locally obtained cane sugar and boiled rainwater. Banana was presented on a plastic dish and replaced twice a week and sugar solution was presented in a small vial mounted on a piece of cardboard, topped off daily and refreshed once every other week. Butterflies were generally observed to find the sugar solution quickly and to feed from the vial, but this was not verified for all individuals. Water was presented as wet tissue

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paper on a plastic dish inside the cages. The tissue paper was moistened by spraying water daily and was replaced weekly.

Differences with the method of Beck (2007) include: no restriction on butterfly wear at capture, use of mashed banana instead of a sugar solution with amino acids, and no salt was provided. By including more worn and thus presumably older individuals, information on aging in the wild could potentially be gained (Carey et al., 2008b; Molleman et al., 2008a; Molleman et al., 2009). Kibale National Park features extraordinary high butterfly population densities (Molleman et al., 2006) that enabled us to obtain our sample sizes in a matter of days for the *Euphaedra* included.

Results

Eight of 117 male butterflies died accidently or escaped from cages and were treated as censored observations. The median life span in captivity of the banana cohorts was insignificantly longer than that of the sugar cohort in all but one species (*C. tiridates*) which had very small sample sizes (7 out of 8 species; Table 1). In *E. medon*, *E. alacris* and the *Charaxes* species combined, banana diet seemed beneficial in the early period of capture but later seemed to reduce survival compared to the sugar diet (Figure 1). To formally test this hypothesis, we pooled all data and constructed a time-varying Cox model to fit the lifespan in captivity (Table 2). Banana diet was compared with sugar using three parameters for three different periods: in the first 35 days in captivity, from the 36th day to the 45th day, and afterwards. Species (with *Charaxes* pooled) were adjusted as a covariate in the model.

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The loss of wing scales, as a surrogate of age at capture (Ehrlich and Gilbert, 1973; Kemp, 2001), was also included as a predictor of life span in captivity. Both 'species' and 'loss of wing scales' satisfied the proportional hazards model assumptions. During the first 35 days in captivity, the butterflies fed sugar had a higher risk of death than those fed banana. More precisely, within this period the instant mortality of a butterfly fed with banana is only 41.4% (exponent of -0.883 reported in table 2) of that of a butterfly fed with sugar (with p-value=0.005 and 95% confidence interval 22.3%, 77.0%), if they belonged to the same species and had the same level of scale loss at capture. During the second period in captivity, the two diet cohorts were not significantly different. After day 45, the butterflies fed banana had lower survival probabilities than those fed sugar (Table 2). A similar experiment on female butterflies found the opposite effect for three species of *Euphaedra*: compared to female butterflies fed sugar, the females fed banana had a lower survival rate at first and survived better after a month in captivity (see Figure 1 for 2 Euphaedra species; Molleman et al., 2009). In contrast to *Euphaedra*, the diet effect was similar for *Charaxes* males and females (Figure 1). Different species also had significantly different male life spans in captivity regardless of diet: *E. medon* lived significantly longer than *E. alacris*, but was not significantly different from the *Charaxes* species pooled (table 2). Within the current sample, we did not find that male butterflies with more scale loss lived significantly shorter (table 2), which was the case for female butterflies of the same species (Molleman et al., 2009). Although the estimated effect size of scale loss was similar (about 1.04) for both sexes, the standard error for that estimate was much higher for males.

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Discussion

Our results corroborate the suggestion of Beck (2007) that nutrients in fruit can phenotypicaly extend life span of male butterflies, although a negative effect on survival was noted later in life. Interestingly, such patterns have not been found in females of *Euphaedra* butterfly species (Molleman et al., 2009), or females and males of the small fruit-feeding butterfly *Bicyclus anynana* (Molleman et al., 2008b). Females of *Charaxes fulvescens* had higher survival rates on sugar based diets than on a (moistened) slice of banana (Molleman et al., 2008a). There are several important caveats for interpreting these results; (1) although we often observed butterflies feeding from the vials with sugar solution we did not verify whether each individual fed from the vial from the first day it entered captivity, while in the banana cohort this issue is not expected to play any role because these butterflies are attracted to the smell of bananas (Molleman et al., 2005c), (2) sticky mashed banana could affect mortality in other ways than through its nutritional value. Because butterflies can be trapped with their wings or other appendages in the sticky mashed banana, sugar fed males may have better survived better later on in captive life; (3) while females were mated and had the opportunity to lay eggs, males in our and Beck's (2007) experiment had no opportunity to mate. In some insect species mating has a male life span cost (i. e. Ferkau and Fischer, 2006; Martin and Hosken, 2004). However, this may not be an important issue here because it is likely that many male butterflies never obtain matings in nature (Beck, 2007; Boggs, 1979), moreover, mating does not confer a life span cost in monarch butterflies (Oberhauser, 1989). The differences between the sexes in *Euphaedra* could be explained by a survival cost of reproduction in females fed banana, not incurred by males.

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Despite these caveats, we have the following evidence that male fruit-feeding butterflies survive better when they feed on a more complete diet; 1) we found large effects in particular for the mixed *Charaxes* group; 2) the results of Beck (2007) in which the first caveat (males ability to find the vial) was avoided; and 3) the opposite diet effects in females of *Euphaedra* species so that we would also need to assume a sex-differential in the ability to find vials with sugar solution in this genus.

Beck (2007) pointed out the implications of his findings for butterfly ecology. More insight into mechanisms may be gained if male-specific hypotheses were developed on resource allocation and life history evolution, and if we had more insight into the cause of death of old captive butterflies. A possible explanation for our finding is a decrease in muscle power in males on poor diets, because this (and possibly decrease in coordination), seems to precede death in insects in captivity. Examples of this include; medflies start to spend time laying on their backs (supine behavior) several days before they die (Papadopoulos et al., 2002); and *Drosophila* with artificially impaired legs appeared to die because the missing of legs decreased their ability to escape from sticky food (Carey et al., 2007).

Muscle mass and power output of insects can increase with adult nutrient intake, or decrease with age or severity of microbial infections, as is well documented in odonates (i. e. Anholt et al., 1991; Marden, 1998; Matsubara et al., 2005; Schilder, 2007). The maintenance (or increase) of muscle power may be aided by nutrients in the adult diet, and in particular amino acids that are important building blocks of muscle proteins. This was found in the damselfly *Calopteryx maculata*, in which males fly away from their stream after emergence to forage without harassment by other males and several

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days later return to the stream after significantly increasing their muscle mass, to then defend territories and obtain matings (Kirkton and Schultz, 2001).

When muscle power plays a role in survival, extension of life span through dietary enrichment could be explained by allocation of nutrients in the adult diet to muscles, or these butterflies having no need to resorb muscles to allocate amino acids to other goals (Karlsson, 1994; Stjernholm and Karlsson, 2000). In contrast, females may allocate these nutrients preferentially to eggs, and as a result life span of females is not extended on a more complete diet. Interestingly, for *Bicyclus anynana*, no such male-female differential response to diet was observed (Molleman et al., 2008b). In this genus, males use pheromones to court females (Nieberding et al., 2007) and produce large spermatophores (but see Ferkau and Fischer, 2006; Molleman et al., 2005a) that not every male is able to produce on consecutive days (Lewis and Wedell, 2007; Molleman et al., 2004). On the other hand, sexual flight dimorphism is slight or absent in this group (Molleman et al., 2005a). The males of *B. anynana* may thus invest more in pheromone and spermatophore production (even though they did not mate in that experiment) than in muscles, resulting in a trade off between life span and reproduction (Boivin et al., 2005; Lewis and Wedell, 2007) similar to females (Molleman et al., 2008b).

In summary, we hypothesize that (a) male reproductive success in these butterflies is largely dependent on muscle power for male-male competition and courtship, so (b) because investment in muscles increases competitive abilities in male-male interactions and in courtship displays as well as ability to evade predators or free themselves from sticky substances, investment in reproduction and longevity is synergistic in these males. Similar principles may apply to other traits that enhance both competitive ability and

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defense against predators, such as horns or antlers in ungulates and canine teeth in primates and pigs. Because males also tend to display risky behavior (e. g. by spending a larger proportion of their time flying in the case of butterflies, or defending their social groups in mammals), the investments in these features may not be associated directly with higher survival in the wild (i. e. Arlet and Isbell, 2009; Moore, 1987).

Amino acids in the adult diet could extend life span by supporting other systems such as immune response (Boggs, 2009; Stoehr, 2007) as well. However, given the differences found between males and females in their survival response, we searched for processes that are more prevalent in or unique to males.

Over all, our data suggest that fruit-feeding provides butterflies with the opportunity to ingest larger quantities of amino acids as adults, and this may have enabled males to evolve mating strategies that require higher amino acid intakes (i. e. intensive patrolling and territorial defense or amino acid enriched spermatophores) over a longer life span. Longer reproductive life spans can be expected to be adaptive for males in these tropical butterfly species because receptive females are available any time (Gotthard et al., 2000) since they have overlapping generations, and females mate multiply and probably in intervals (Molleman et al., 2005a).

Further experiments that (1) use sugar solution based diets (as in Beck 2007), (2) include verification of feeding of each individual, (3) include longitudinal (time series of individuals) mass (and if possible muscle mass) and activity measures, and (4) explore dietary-restriction gradients (Carey et al., 2008a; Lee et al., 2008; Maklakov et al., 2008; Skorupa et al., 2008), are needed to provide more definite answers on how nutrients affect male survival patterns in captivity. In addition, the results for *Charaxes* need

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further verification because the pooling of data from different species was statistically convenient, but possibly not biologically appropriate. The crucial question of how representative such captive studies are for life in the wild might then be approached by measuring the nutritional and muscular state of butterflies in the wild using mark-recapture or captive cohort protocols. This may be particularly important for these tropical butterflies because the keeping conditions in cages may be poor, or they need much space to exert their agile life-style and some species had to be excluded from my captive studies for this reason (Kelson, 2008; Molleman et al., 2005b; Molleman et al., 2007). In addition, Beck and Fiedler (2009) found a large discrepancy between the life span data from captive and field-studies and assumed that poor keeping conditions in caged tropical studies may be responsible for this finding.

That wing-damage was a not a significant predictor of remaining lifespan in captivity may indicate that rating butterfly age by wear is not so reliable in these species (see for further discussion Molleman et al., 2008a; Molleman et al., 2009).

Our results indicate that nutrient allocation strategies of males and females of the same species can differ more from each other, than from individuals of the same sex of distantly related species. Even though male insects are not considered important in demography and do not provide the advantage of convenient longitudinal measurement of an important aspect of reproductive success (egg production), they merit further investigation. In particular, studies on males can aid in gaining a better understanding of life history evolution because they display a wider variety of tactics for obtaining reproductive success. Our results further challenge the generality of dietary restriction as a way to extend life span in animals.

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Tables:

Table 1: Summary statistics for male butterflies collected from Kibale Forest, Uganda with; sample sizes (number of censored observations in brackets), median life span in captivity in days and maximum life span. Data for con-specific females are given in italics for comparison but statistical details for females are treated elsewhere. The longest median and maximum life span for each species and sex is in bold. As banana fed males had higher survival rates during the first 35 days, median lifespan tended to be longer for the banana cohorts, but as older (>45 days) males showed the opposite effect, sugar fed males often had the longest captive life span for a species.

	N (censored)		Median Life Span		Maximum Life Span	
Species	sugar	banana	sugar	banana	sugar	Banana
E. medon ♂	17 (4)	20 (2)	26	**44**	**123**	86
♀	*18 (4)*	*21*	*31.5*	***39***	*59*	***80***
E. alacris ♂	23 (2)	20	22	**39**	64	**112**
♀	*19 (3)*	*23 (1)*	*29*	***32***	*50*	***83***
Charaxes comb. ♂	22	15	26	**40**	**108**	62
♀	*12 (2)*	*14*	*10*	***30***	***76***	*52*
C. numenes ♂	6	5	32.5	**33**	**83**	40
♀	*6 (1)*	*5*	*21.5*	***33***	***76***	*43*
C. bipunctatus ♂	3	1	27	**58**	**108**	58
♀	*2 (1)*	*3*	*3*	***39***	***3***	***52***
C. tiridates ♂	2	1	**47**	43	**52**	43
♀	*0*	*2*		*10.5*		*15*
C. protoclea ♂	5	4	25	**43.5**	51	**62**
♀	*2*	*2*	*22.5*	***26.5***	***40***	*30*
C. Cynthia ♂	4	2	22.5	**25.5**	**63**	45
♀	*1*	*0*	*45*		***45***	
C. pollux ♂	2	2	13	**43**	22	**50**
♀	*1*	*2*	*4*	***36***	*4*	***42***

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Table 2: Parameter estimation in the time-varying Cox model with *E. medon* at zero scale loss as the baseline. Results are significant when the confidence interval does not contain 1. Note that time effects vary among species and are most pronounced in the pooled *Charaxes* species. When *Charaxes* are excluded, the <35days period still shows a significant diet effect, but the >45 days is no longer significant.

Variable	Parameter Estimation	Standard Error	p-value	Hazard Ratio	95% CI for Hazard Ratio
Diet <35 day	**-0.883**	**0.317**	**0.005**	**0.414**	**[0.222, 0.769]**
Diet36~45 day	0.206	0.379	0.586	1.299	[0.585, 2.581]
Diet >45 day	**0.769**	**0.378**	**0.042**	**2.157**	**[1.028, 4.525]**
Charaxes	0.384	0.250	0.124	1.468	[0.900, 2.395]
E. alacris	**0.568**	**0.248**	**0.022**	**1.765**	**[1.085, 2.869]**
Scale loss	0.035	0.026	0.188	1.035	[0.983, 1.090]

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Figure:

Figure 1:

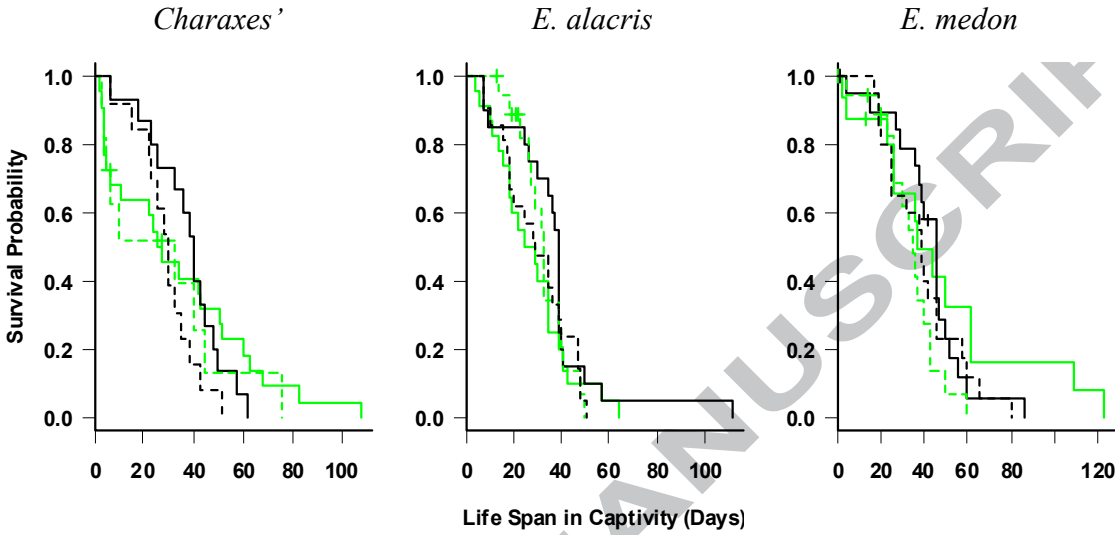

Figure 1. Kaplan-Meier Estimation of Survival Probability of a mix of large *Charaxes* species and two *Euphaedra* species. Life span of males are presented as solid lines, female butterflies are represented by dashed lines. The grey/green is for sugar and black is for banana diets. Note that the survival probability here is not adjusted for other covariates such as scale loss.

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