UNIVERSITY OF CALIFORNIA,
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Evolutionary Biology of Diet, Aging, and Mismatch

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Grant Allen Rutledge

Dissertation Committee:
Professor Michael R. Rose, Chair
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DEDICATION

To

My parents Gunilla and Steve and my sisters Jennifer and Marlaina
For your unconditional love.

To

My advisor Dr. Michael R. Rose

Whose passion for science and teaching inspires me every day.

And to

The undergraduate researchers of the Rose and Mueller Labs

For your hard work.

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PRESENTATIONS

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ABSTRACT OF THE DISSERTATION

Evolutionary Biology of Diet, Aging, and Mismatch

By

Grant Allen Rutledge

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2018

Professor Michael R. Rose, Chair

The search for a diet or supplement that improves healthspan in humans has been daunting. This dissertation applies theory on the evolution of aging to understand what diets are best for extending healthspan. Chapter 1 reviews strategies for healthspan extension using diet and supplementation from the standpoint of evolutionary biology. Then it introduces a new evolutionary strategy for dietary enhancement of healthspan. Our intuitive understanding of adaptation by natural selection is dominated by the power of selection at early ages in large populations. Yet, as the forces of natural selection fall with adult age, we expect adaptation to be attenuated with age. Explicit simulations of age-dependent adaptation suggest that populations adapt to a novel environment quickly at early ages, but only slowly and incompletely at later adult ages. Chapter 2 tests for age-dependent adaptation in laboratory populations of *Drosophila melanogaster*. The results show clear age-specificity of adaptation in two examples of evolution in response to dietary transition. In the first example, populations perform better on an ancestral, long-abandoned, fruit diet compared to an evolutionarily recent fruit diet, only at later ages. In the second example, the gain and loss of urea adaptation is strongest at early ages and weakest at later ages. Chapter 3 evaluates the effects of combining both diet and botanical supplementation on

Drosophila healthspan. One botanical extract, derived from *Rosa damascena*, decreases survivorship when added to a long-abandoned, ancestral diet, but increases lifespan when added to an evolutionarily recent diet. Another botanical, derived from *Rhodiola rosea,* extends life-span by approximately 20% with a 20% decrease in average fecundity. In addition, there is evidence of an antagonistic effect when these botanicals are combined, supporting the "Poisoned Chalice" hypothesis that novel combinations of substances may produce adverse physiological responses. Chapter 4 studies the effects of evolutionary history on phenotypic convergence in *D. melanogaster* populations selected for desiccation resistance and larval urea tolerance. We find that extreme selection can have long-lasting impacts on phenotypic differentiation, particularly for longevity, even after a few hundred generations of relaxed selection.

CHAPTER 1

An evolutionary analysis of healthspan extension using diet[1]

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ABSTRACT

The search for compounds that enhance healthspan has been daunting. Many gerontological experiments on model organisms, including *Drosophila* species, have examined the effects of individual substances on lifespan solely. But it is now clear that effective alleviation of aging requires more than merely prolonged survival regardless of other functional effects. Monitoring other life-history characters is imperative. In addition, functional characters such as locomotor and cognitive capacities may be important too. Here we review the topic of healthspan extension using diet from the standpoint of evolutionary biology. We discuss proposed "rules" for evaluating candidate anti-aging substances. We point out the failings of some studies of anti-aging substances, such as resveratrol. We also critically review proposed anti-aging strategies that have been based on evolutionary reasoning, questioning some of our own earlier suggestions. Here we offer a new evolutionary strategy for dietary enhancement of healthspan, one that is as applicable to fruit flies as humans. However, our overall view is that the project of ameliorating aging using ingestible substances is without doubt challenging to a high degree.

Literature Review

The Ponce de Leon Trail Problem: Looking for Anti-Aging Substances

A. The Ponce de Leon Trail is Very Old.

One of the more universal features of the historical record of biological research is the search for a substance that can postpone or reverse the effects of aging on people. This is a ubiquitous topic in Taoist writings (vid. Needham's *Science and Civilization in China* books, 1954-2008), and a commonplace theme of Traditional Chinese Medicine, which grew out of Taoist traditions. In the West, the topic was of interest in ancient civilizations, as illustrated by the legend of Gilgamesh from Sumerian civilization.

After Western civilization recovered from the Dark Ages and Middle Ages millennium of hostility to biological research, the topic resurfaced in the work of the Renaissance alchemists, such as Paracelsus. Perhaps the most famous early-modern Western example of this search for a restorative substance is the possibly apocryphal story of Ponce de Leon looking for a fountain of youth in Florida, after the voyages of Columbus to the New World (Haycock, 2009). Of greater significance for academic biology, the polymath and founding figure of Western science Francis Bacon devoted an entire book to the topic of aging and how it can be influenced, *Historia Vitae et Mortis* (1637). But because Francis Bacon was by inclination and prescription skeptical, it is more appropriate to refer to the project of controlling aging by means of substances as the Ponce de Leon Trail (cf. Moment, 1978).

For a *very* long time by the standard of contemporary biology, the genus *Drosophila* has been a model system of choice for the control of aging. For example, Loeb and Northrop (1917) used temperature to control rates of demographic aging in laboratory fruit flies almost a century ago, a practice that has continued ever since (e.g. McArthur and Sohal, 1982). This fruit fly aging

literature has increased explosively, with hundreds of publications claiming to demonstrate the experimental manipulation of *Drosophila* aging using ingested substances and other interventions. This is a literature too vast to be enumeratively reviewed. Instead, what we offer here is a critique from the standpoint of evolutionary biology.

B. We need to study Healthspan.

A central point for us, to begin with, is the importance of what is sometimes called "healthspan." Crudely speaking, this can be thought of as a combination of the capacity to survive together with a capacity, or capacities, to function. That is to say, to take an extreme example we do not regard the prolongation of human life in a medically-induced coma as a notable achievement of anti-aging. Effective mitigation of aging should be more than merely prolonged survival.

Fortunately, in the context of evolutionary theory there are well defined quantitative measures of healthspan that can be used for objective experimentation. One such measure would be R_o, which is the summation over all ages of the products of survival probability to a particular age with the fecundity at that age (vid. Charlesworth and Charlesworth, 1973). [Similar measures would include the survival probability *at* a particular age multiplied by the fecundity at that age, summed over all ages.] The point of such measures is that the central function of living things, from the standpoint of Darwinian theory, is reproduction. In effect, everything else about life-history is subservient to that end, with the appropriate modifications for inclusive fitness when there are significant transfers of resources between individuals, such as occur with parental care (vid. Hamilton, 1964a,b; Lee, 2003). Thus, it is more than just appropriate to use such summations of survival probabilities and fecundities to calculate total healthspan. We would argue that such indices provide correct scientific measures of healthspan. However, for the present purpose we

need only argue that some measure of healthspan of this kind is necessary for the measurement of net effects on aging, properly considered as a whole.

C. Rules for Studying Anti-Aging Candidate Substances.

A useful starting point for the study of the healthspan effects of ingested substances was supplied by Jafari and Rose (2006), which proposed a set of rules for the design of model organism tests of candidate anti-aging substances. One of their starting points, which we share, is the demographic partitioning of life-history into three phases: development, aging, and late life (cf. Mueller et al., 2011). Though McCay's classic experiments on dietary restriction in rodents incorporated lifespan extension arising from either protracted developed or prolonged adult survival (vid. McCay et al., 1939), almost all gerontologists since then have agreed on the point that useful anti-aging trials should focus on adult life, after the completion of development.

What is still controversial is the status of late life in anti-aging experiments. Late life is a distinctive phase of life first well-characterized from human demographic data by Greenwood and Irwin (1939) as a plateauing in mortality rates at very late ages, after the age of 90 years in their data. However, the phenomenon of late-life mortality rate plateaus was not generally credited as a significant biological phenomenon until the publications of Carey et al. (1992) and Curtsinger et al. (1992), which used laboratory medflies and *Drosophila melanogaster,* respectively. Rauser et al. (2003, 2005, 2006) subsequently demonstrated a comparable, though not synchronous, plateau in later-life fecundity. Within evolutionary genetic theory, the selective pressures that characterize aging and late life phases are qualitatively different (e.g. Mueller and Rose, 1996; Charlesworth, 2001; Mueller et al., 2011), which is how evolutionary biologists like ourselves explain the distinctly different demographic patterns of these two parts of adult life-history.

Jafari and Rose (2006) suggested that experimental trials using *Drosophila* should study the effects of candidate anti-aging substances on mortality rate during the aging phase of life only. This is an elegant solution to the quantitative complexity of the full adult life-cycle. However, if we are successful at slowing human aging demographically, many more people will survive to reach late life than were found to do so by Greenwood and Irwin (1939). This makes the impact of candidate anti-aging substances on the post-aging late-life phase also of interest. However, what is indubitable is that there is potential for significant confusion about the impact of a candidate anti-aging substance *if* the demographic analysis of its effects does *not* consider the existence of post-aging adults in an experimental cohort of fruit flies. Unlike the human case at present, some *Drosophila* laboratory cohorts have many individuals surviving into late life (e.g. Shahrestani et al., 2012), much as found by Carey et al. (1992) for medflies. Overall then, we are more agnostic than Jafari and Rose (2006) about the advisability of confining the study of the effects of candidate anti-aging substances to the aging demographic phase only.

A classic concern of pharmacologists like Jafari (vid. Jafari et al., 2007a,b) is that one cannot be sure that the candidate substance, rather than some artifact, is having the inferred anti-aging effect unless there is a dose-dependent pattern to the response. That is, the healthspan effects of a candidate anti-aging substance should scale with the dose. Again, we have some qualifications that we will apply to this stricture from Jafari and Rose (2006), particularly where qualitative changes in diet are concerned.

Jafari and Rose (2006) further contended that experimental *Drosophila* that are being used in a test of a candidate aging substance should not be hypometabolic. As humans are homeotherms with fairly stable metabolic rates, drugs and other interventions that act via gross lowering of metabolic rates in poikilotherms like fruit flies, producing a state of hypometabolism, are not

appropriate candidates for anti-aging interventions among human subjects. This was patently the case in the work of Loeb and Northrop (1917), and it is a well-known phenomenon in experimental physiology. In Djawdan et al. (1996), no differences in metabolic rate were observed between the experimentally evolved longer-lived and shorter-lived flies of Rose (1984), and that was a material point in the case for the value of those *Drosophila* for aging research (vid. Rose et al., 2004). Therefore, a drug that increases lifespan at the expense of a decrease in metabolism is not an ideal candidate anti-aging substance for adoption by humans.

In the same vein, Jafari and Rose (2006) argue that candidate anti-aging substances should not curtail fecundity. It has been well established that lowering fecundity in fruit flies can dramatically increase longevity, for example by dietary restriction (e.g. Chippindale et al., 1993), but also when fecundity is depressed by other means (e.g. Maynard Smith, 1958). Compounds that substantially lower fecundity may increase longevity from reduced 'cost of reproduction' effects alone. Again, a key point is that gross depression of total fecundity is not associated with evolutionarily postponed aging (Rose, 1984; Leroi et al., 1994; Rose et al., 2004). However, in the framework that we are developing, measures like R_o naturally take depressed fecundity into account, so this problem in effect washes out in the quantitative measures that we recommend.

Following the same line of argument, Jafari and Rose (2006) emphasize that experimental model organisms should not have general nervous system impairment as a result of a candidate substance. A typical example of such an effect can be achieved by a general-purpose tranquilizing substance. But again, appropriate healthspan measurement should directly obviate this problem, in that heavily tranquilized fruit flies are not going to be mating, feeding, or reproducing at a normal rate.

Elixirs of Life? Single substances have a problematic record in the Drosophila aging literature

A. Why healthspan studies must consider reproduction

Fruit flies are increasingly being used to test candidate pharmaceuticals for long-term health benefits. There are many anti-aging studies of *Drosophila* supplementation with a wide variety of substances, from antioxidants such as resveratrol and lipoic acid to histone deacetylase inhibitors like phenyl butyrate (e.g. Bauer et al., 2004; Kang et al. 2002). However, Matsagas et al. (2009) demonstrate that some single substances have ostensibly beneficial effects when only longevity or mortality rates are monitored, effects that might be an artifact of functional impairment of reproductive characters.

An example of this problem is provided by the study of Bahadorani et al. (2008). Vitamins A, C, and E are each thought to play an important role in mitigating oxidative stress. Accordingly, each was administered to *Drosophila* cohorts under oxidative stress conditions. Under chronic oxidative stress conditions, some of these supplements increased lifespan and some decreased lifespan. However, only lifespan was measured in this study. Bonilla et al. (2002) is another example of pharmaceutical supplementation research studying only fruit fly lifespan effects. Melatonin, a hormone that is thought to prevent oxidative damage to fly tissues, was added to nutritional medium. Lifespan was significantly increased from 61.2 days in the controls to 81.5 days in the melatonin-fed flies. Once again only lifespan was observed in this study.

As a general rule, effects on reproduction and other functional characters are often not measured in fruit fly drug studies that measure survival rates or longevity. Yet decades of genetic and manipulative *Drosophila* research have shown that longevity is just one part of the spectrum of life-history characters that jointly respond when fruit fly longevity is impacted significantly.

Thus, average longevity on its own may be a poor measure of the full spectrum of effects of administered substances. In other words, most *Drosophila* studies of the effects of dietary substances fail to adequately document the range of healthspan effects.

Although not intentionally achieved by supplementation with pharmaceutical substances, dietary restriction (DR) in model organisms like *Drosophila* is well-known in animal cohorts to increase average lifespan in conjunction with reduced fertility (e.g. Chippindale et al., 1993; Chippindale et al., 1997). Figure 1.1 demonstrates hypothetical results of DR fly studies that monitor *both* survivorship and fecundity. Chippindale at al. (1993) performed a series of experiments in which the amount of live yeast inoculate applied to the substrate was varied. Lower yeast levels, which significantly reduce fecundity, enhanced longevity. However, it is also important to note that an overly-extreme reduction of food levels will lead to a reduction in lifespan and fecundity.

Chronic exposure of an experimental cohort to a pharmaceutical drug could have a superficially beneficial effect if it reduces nutritional intake due to the flies' perceived noxiousness of the drug for the model organism. Also, an animal may be sickened to the point of lethargy by a substance, even if its feeding rate is not reduced – such as would be the case with an addictive opiate analog, leading to reduced reproduction.

Research with urea supplementation in adult *Drosophila* provides a clear example of toxicity-induced increase in life-span. Joshi et al. (1996) and Santos et al. (in prep.) demonstrate that when adult *D. melanogaster* are maintained on food supplemented with urea, longevity of both males and females is significantly increased. In addition, female flies maintained on urea-supplemented food exhibit a consistent decline in fecundity over time, relative to those maintained on regular food (Figure 1.2a) (Joshi et al. 1996; Santos in prep.). The toxicity of urea is apparent

when you expose larva to it: there is a significant decrease in mean adult longevity and an increase in age-specific mortality. Female flies exhibit a dramatic decrease in fecundity as a result of exposure to urea as larvae too. Figure 1.2b graphically instantiates this using a healthspan measure known as "$p_x m_x$" [product of female conditional survivorship (p_x) and eggs per surviving female (m_x)] (Chapter 2).

B. Single-substance failures of replication

A key strategy in the publication of "successful" single-substance interventions is to (1) avoid collecting adverse healthspan information, (2) avoid detecting adverse healthspan side-effects by using inadequate replication or technique, or (3) suppress/fail to publish any such adverse results if they have been obtained. Usually tactic (3) is not necessary, because biologists can be expert at avoiding the collection of data that would impinge on the "story" that they want to tell. We have ourselves been involved in collaborations where our (now former) colleagues have suppressed results that were adverse to their favored thesis.

As an example of practices that are at least less than ideal, we have the polyphenol resveratrol, a natural compound found in commonly-consumed plants and notoriously present in red wine. Resveratrol has received much attention in scientific studies (Howitz et al., 2003; Valenzano et al., 2006; Lagouge et al., 2006; Baur et al., 2006; Morselli et al., 2010; Miller et al., 2011). However, the lifespan results have been variable. Resveratrol is thought to be a sirtunin2-activating antioxidant compound (Wood et al., 2004; Bauer et al., 2004). The authors of some studies have suggested that resveratrol acts as a caloric restriction mimetic due to general sirtuin activation (Howitz et al., 2003; Wood et al., 2004). However, Kaeberlein et. al (2005) found that resveratrol has no detectable effect on Sir2 activity *in vivo* or on life span in yeast. On the other hand, resveratrol has been shown to increase lifespan in *Drosophila* studies with little or no

obvious effects on fecundity as shown in Figure 1.3a (Wood et al. 2004). Yet Bass et. al (2007) found no significant effects of resveratrol on lifespan in seven independent trails, three of which used the same strain as was used in Wood et al. (2004) (Figure 1.3b).

Bass et al. (2007) was not able to reproduce the longevity increasing property of resveratrol on the *D. melanogaster* strain Canton S in three of their trials, and they also did not obtain positive results using the strain Dahomey. One possible explanation for such inconsistent results is that the effect of a candidate anti-aging substance can be dependent on the genetic ancestry of the cohort(s) undergoing pharmacological trials. It is a well- established principle of epistasis that some genetic backgrounds will respond differently to the introduction of the same mutation. In the case of anti-aging drug trials, it is possible that a compound might increase lifespan in a stock that has accidentally fixed a particular gene, or set of genes, yet the same compound given to a different stock of fruit flies might have no effect on healthspan. In such cases, it would be fair to say that the impact of the candidate anti-aging compound depends critically on the genetics of *D. melanogaster*, making its general value dubious.

Kang et al. (2002) reported that feeding *Drosophila* with 4-phenylbutyrate (PBA) can significantly increase lifespan without a reduction in other healthspan characteristics like reproductive ability. However, Jafari et al. (2006) pointed out that 10 mM of PBA resulted in lifespan extension in a *white* mutant strain while the wild type strain only required 5 mM of PBA for lifespan extension.

Another possible cause of ambiguous or inconsistent results from single-substance trials could be effects on metabolic rate and locomotion. Avanesian et al. (2010) tested the common anticonvulsant Lamotrigine. It was hypothesized that this chemical increased lifespan at the expense of decreasing healthspan. They found that lamotrigine did in fact increase lifespan,

however a reduction in locomotor activity and metabolic rate depression were also observed. Matsagas et. al (2009) performed experiments testing the effect of sedatives on lifespan and healthspan of *Drosophila*. Lithium, a commonly used sedative, slightly elevated mean longevity at the two lowest doses. However, there was a significant negative impact on fecundity and male mating success even at those doses.

Another possible cause of difficulties with single-substance trials arises when the effect of a medication is highly sensitive to the fly culture environment. Among other things, it is possible that recondite environmental effects on longevity are fairly minor, but the effects on fecundity or mating behavior are much greater. Under these conditions, some anti-aging medications may have a beneficial direct effect on adult survival, but inconsistent deleterious side-effects which are difficult to control or to resolve, especially if no data are collected on the effects of the substance on the other life-history characters. It is at least conceivable that the inconsistent results observed with resveratrol are due to poorly controlled recondite effects on other life-history characters, such as female fecundity. Wood et al. (2004) tested the effect of resveratrol in a low-calorie environment (Figure 1.3 left dotted lines) and determined that no significant increase in lifespan was observed. They concluded that the lack of a response in the DR environment suggested that resveratrol must extend lifespan through some mechanism that is related to caloric restriction. We would suggest that, given the difficulty of reproducing an anti-aging effect of resveratrol, variable secondary life-historical effects could be obscuring its general impact on healthspan. However, pharmacological anti-aging effects that are not robust over trials that have a range of culture conditions suggest that such drug treatments may not have the consistency that would warrant their further study for medical applications. Table 1.1 summarizes various longevity-increasing compounds and their possible adverse effects on healthspan.

Hamiltonian and Genomic Approaches to Healthspan Manipulation

A. *Hamiltonian gerontology vs. aging as cumulative damage*

The common assumption among many gerontologists, particularly those that do not study aging from an evolutionary perspective, is that aging is a process of accumulating damage. With age, it is supposed that organisms accumulate damage through oxidation, free radicals and the like. It is doubtful that significant progress will be made in the manipulation of aging with these presuppositions.

Bluntly put, the falsity of conventional *damage* theories of aging is well demonstrated by the following facts. (1) There are fissile organisms that show no detectable aging, both unicellular and multicellular (Finch, 1990; Rose, 1991). (2) Non-fissile species with ovigerous reproduction nonetheless are sustained by unbroken cell lineages that are hundreds of millions of years old, whether these lineages engage in sex or not. (3) Aging in some laboratory cohorts of sufficient size comes to a halt at later ages, as discussed previously. Together these findings falsify any theory of aging that is based on a universal process of cumulative damage akin the Second Law of Thermodynamics.

Aging is instead due to the declines in the Hamilton's Forces of natural selection which occur at the start of adult life in species with clear separation of the products of reproduction from the adult soma (Hamilton 1966; Charlesworth 1980; Rose 1991; Rose et al. 2007). Because natural selection produces adaptation, as the power of natural selection declines, a decline in adaptation with age is expected. Evolutionary biologists have further been able to readily and substantially postpone fruit fly aging by manipulating Hamilton's Forces (Rose and Charlesworth 1980; Luckinbill et al. 1984; Rose 1991; Rose et al., 2004), a track record that is unmatched by attempts to manipulate aging based on non-evolutionary gerontological theories such as those based on

cumulative damage. This leads us to conclude that Hamiltonian gerontology, as outlined in Rose (1991) and developed further in Mueller et al. (2011), delivers the best scientific foundation on which to design or evaluate attempts to intervene in aging.

Rose et al. (2010) addresses at length the question of how to develop Hamiltonian strategies with which to ameliorate human aging. The strategies that they discuss are based on starting with organisms that have had their aging slowed by manipulating Hamilton's forces of natural selection and then reverse engineering the biology of those longer-lived organisms to discover interventions that can be used to ameliorate aging in other organisms, including humans. The so-called "Methuselah Flies" that have evolved slower or delayed aging (Rose et al. 2004) are readily available sources of physiological and genomic information with which to find candidate substances that might ameliorate healthspan. In particular, these flies have also been shown to have greater (i) stress resistance, (ii) total reproductive output, and (iii) athletic capacity (Rose et al., 2004). Thus these are not flies that have achieved greater lifespan as a result of reduced overall reproductive output; rather, they have massively extended healthspan.

B. Finding which genes to target with pharmaceuticals or nutritional substances

As explained in Rose et al. (2010), in 2006 Rose and colleagues compared whole-genome gene-expression patterns in Methuselah Flies with their matched controls. They found about 1,000 genes showing statistically consistent differences in expression. These genes are presumptive indicators of the genetic changes that underlie the substantially ameliorated aging achieved using Hamiltonian methods in fruit flies. Seven hundred of these genes had matching orthologous loci in the human genome and about 100 of the 700 human genes were considered candidate pathways to target to slow aging in *both* fruit flies and humans based on parallel findings from genomic analysis in the two species.

But that early gene-expression analysis was only a first step toward the genomic analysis of the genetic foundations of Hamiltonian healthspan extension. Re-sequencing studies in *Drosophila* have shown that experimentally evolved differences in aging involve SNP frequency changes at hundreds of locations across the fruit fly genome (Burke et al., 2010; Rose and Burke, 2011). Because of this, it will be very difficult, if not impossible, to find effective anti-aging pharmaceutical agents that increase healthspan by targeting a single pathway.

C. Can we use multiple supplements to slow aging?

If aging is due to just a few "master regulatory genes" (vid. Guarente and Kenyon 2000) or a small number of types of accumulating damage (e.g. de Grey and Rae 2007), then we can suppose that massively effective "anti-aging" supplements containing just a few substances might be discovered. Radically successful anti-aging formulations would then only have to target those few genes or stop a few pathways of accumulating damage. But all the experimental evidence on this point suggests instead that aging is rarely, and perhaps never, due to just a few master regulatory genes.

From a Hamiltonian perspective, it is clear that in order to slow aging with substances, we will need to retune hundreds of genetically defined mechanisms of aging. Natural selection can do this for us as Methuselah Flies demonstrates. It will be very hard to get a small number of powerful pharmaceuticals to do this, but numerous substances of individually small effect conceivably might. Thus Rose et al. (2010) propose that the best strategy to emulate the effects of natural selection in extending lifespan might be nutritional supplementation with many supplements that individually have physiological effects of small magnitude.

But this does not necessarily mean ingesting the hundreds of supplements that many modern-day molecular biologists and physicians recommend. [In fact, we predict a failure of such

supplementation to produce extended human healthspans, for reasons we will discuss later.] The Hamiltonian perspective suggests using nutritional supplements in the same manner as evolution often uses genetic variants of small effect. Rose et al. (2010) proposed screening candidate "nutrigenomic agents" for small to moderate benefits, just as natural selection screens new genetic variants for their beneficial effects. In the case of genomically-informed substance testing, the experimenter can choose candidate substances based on biochemical information about the effects of candidate substances on the specific pathways genomic analysis of healthspan extension has identified. This seems like a plausible strategy. However, we will suggest here that it may face potentially fatal challenges.

The Poisoned Chalice Problem: Do Animals Perceive Too Many Novel Substances as Poison?

There is a widespread belief that supplementing our diets with large amounts of isolated nutrients or vitamins will enhance healthspan, a belief that motivates many thousands of people to take a plethora of supplements that have one or another claimed or merely conjectured health benefit (vid. Kurzweil and Grossman, 2004; 2009). Perhaps because of the universal failure to find a single Ponce de Leon substance that provides everlasting youth in fruit flies, mice, or humans, the present-day hope is to combine hundreds of substances for a net enhancement of healthspan. The Hamiltonian strategy of Rose et al. (2010) is no exception to this general ambition. It seems plausible that if you have many single-substance successes, one could combine these substances and in sum propitiously compound life-extending properties.

Unfortunately, studies of both humans and mice have not found that combined supplementation is more successful than supplementation with single substances. For example, Macpherson et al. (2013) performed a meta-analysis of randomized controlled studies in humans.

Across all studies, no effect of multivitamin treatment on all-cause mortality was seen. Furthermore, cohort studies of human multivitamin use and mortality have found no benefit (Watkins et al., 2000; Park et al., 2011). Among the diverse studies of multifold supplementation, it is clear that those of Spindler (e.g. 2012; 2014) have achieved the highest standards of design and replication. Spindler et al. (2014) performed isocaloric studies in mice to test the hypothesis that complex mixtures of dietary supplements including vitamins, phytochemicals, and other nutraceuticals could increase the longevity of initially healthy mammals. In addition, nutraceutical, vitamin, or mineral combinations that have had success in previous studies were tested again. Spindler et al. (2014) found that there was no significant increase in rodent lifespan for any supplement mixture *including combinations that had been reported to increase lifespan in previous experiments*. Also, some of the more complex mixtures tested significantly decreased lifespan.

We have an evolutionary hypothesis that we would like to offer to explain these experimental results. We also suggest that this hypothesis provides a cautionary note even for the Hamiltonian and genomic strategies advocated by Rose et al. (2010). We call this the "poisoned chalice" hypothesis.

Metazoa are not Erlenmeyer flasks. That is, our bodies are not inert vessels in which numerous parallel biochemical reactions occur independently of each other. Instead, natural selection has created enormous "kluged" networks of physiology that collectively enhance our Darwinian fitness, often by incorporating bits and pieces of molecular machinery that act both summatively and sometimes in antagonism with each other. Furthermore, this complex large-scale interacting network has feedback circuits that respond to features of the environment, much like

control-theory designed stabilizing components of complex electronics function to sustain circuit signaling integrity and to prevent destructive overload of circuits.

Thus, in the case of *Drosophila,* we know that flies actively modify their physiological functioning in the event of elevated temperatures, the so-called "heat shock" response. Likewise, acute starvation abruptly modifies reproductive activity (Chippindale et al., 1993), which is a response that is implicated in the physiological machinery underlying the extension of longevity in conjunction with the decrease in reproduction observed in dietary restriction. Likewise, exposure to urea elicits an abrupt reduction in reproduction, which may at least partly explain the resulting extension in lifespan, as we have discussed here.

Perhaps the provision of many novel substances to humans, mice, or fruit flies elicits the same kind of physiological responses as those elicited by urea exposure or acute starvation? That is, in the specific case of *Drosophila,* when fruit flies are exposed to culture medium that this is so novel that their physiology reacts as if they are in an environment which is suboptimal for reproduction, they may shut down functional components of their aggregate physiology. Such "shut downs" may reduce reproduction, or they may curtail activity through sedation, or they may indeed curtail cellular repair processes vital to organismal survival. Likewise, we would suggest, assaulting human physiology with numerous substances that our digestive machinery and other pieces of our metabolic machinery react to as low-grade poisons may trigger toxicity reactions. The net effect of too many of these toxicity reactions may be to reduce overall healthspan, not increase it.

In effect, we suggest, the provision of multiple, purified, novel substances of individually small effect may result in a supplementation cocktail that is a poisoned chalice for the kind of metabolic machinery that differentiated multicellular animals possess. In a phrase, almost all such

complex supplementation regimens may amount to a poisoned chalice for healthspan, not some elixir of life.

As an alternative, we suggest, what is needed is dietary intervention that our physiologies unequivocally "accept" as healthy food, forestalling any adverse poisoned-chalice reaction. The question then becomes, what would such an optimal food be like? How can we find such an ideal dietary regime?

Is There a Hamiltonian Holy Grail for Human Healthspan Extension?
Going backward in evolutionary time as you go forward in biological time

Recently we had another idea of some relevance for the discovery of better diets based on evolutionary biology. We developed this idea from considering the evolution of a population that has undergone a substantial change of diet in recent evolutionary time. The evidence we have from experimental evolution suggests rapid adaptation to a novel environment (e.g. Matos et al. 2000), particularly for early components of fitness such as developmental speed and initial fecundity. But Hamilton's forces of natural selection fall with adult age in almost all cases, which should produce weaker adaptation at later ages in the first generations after dietary change (Mueller et al., 2011). Explicit simulations of this have the expected effect: lack of adaptation to the new environment at later ages (Phung et al., in prep.).

We have experimentally tested this idea in our *D. melanogaster* lab populations, because they have undergone a major change in diet since their introduction to the laboratory in 1975, approximately 1,000 generations ago. As expected from this age-dependent effect on adaptation, at later ages our flies are better adapted to a crude approximation of their ancestral diet in the wild (Chapter 1) (Figure 1.4). So the idea works in explicit theory and in careful laboratory experiments. Its practical application is that older humans might be able to improve their healthspans by switching to diets that resemble those of our Paleolithic ancestors, given that our adoption of the

agricultural Neolithic diet is relatively recent in evolutionary terms, about 200-400 generations ago.

But the more general principle that this line of research suggests is that there indeed *are* complex dietary changes that can be made which will enhance our healthspans. In the particular case of our fruit flies given their ancestral diet from more than 1,000 generations ago, we have stumbled in the direction of such a dietary change based on our knowledge of their evolutionary history, at least over the last few hundred years. This supports the general ambition to provide improved diets for human healthspans based on evolutionary insights.

What remains an entirely unanswered question is whether or not we can ever do better, for fruit flies or humans, than the adoption of the diet that evolution long ago tuned our physiologies to exploit efficiently. We can of course more effectively home in on what evolution has already achieved, by learning more about the details of fruit fly or human evolutionary histories. But can we do even better than to exploit what evolution has already accomplished, with respect to the creation of a maximal healthspan? That remains a tantalizing question for which we have no answer at present.

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FIGURES

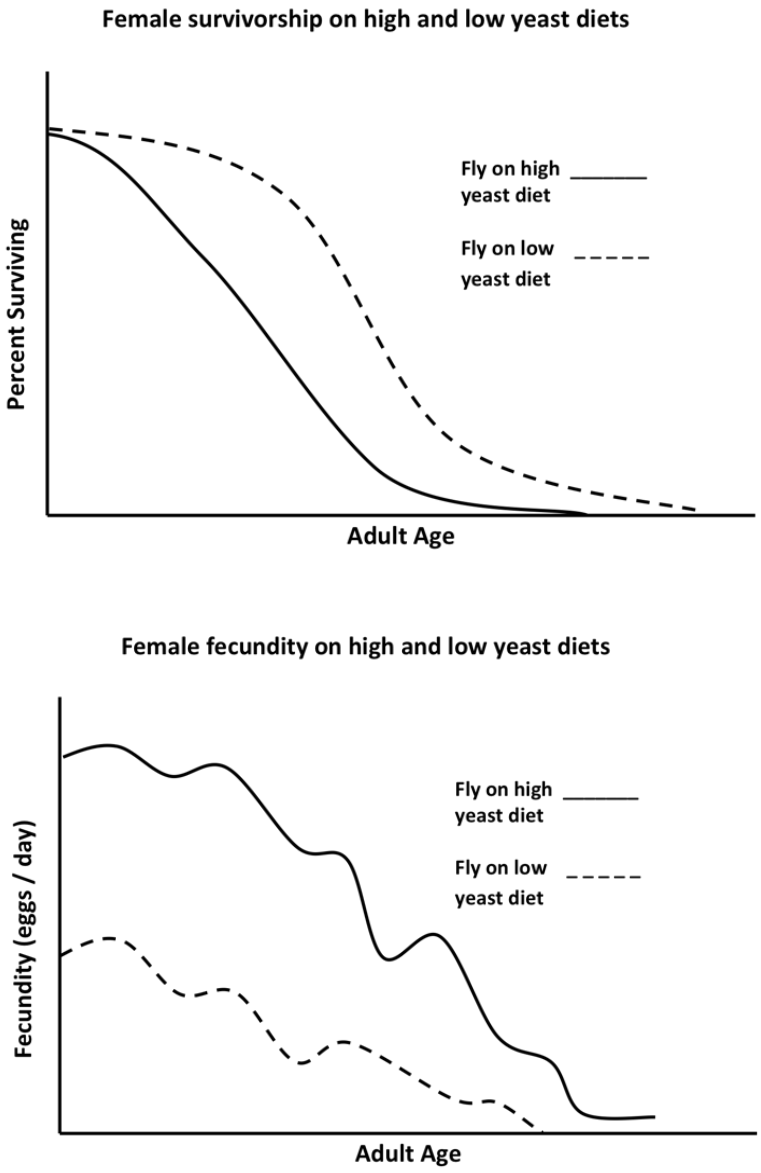

Figure 1.1 Hypothetical effects of dietary restriction on *Drosophila* using high and low levels of yeast inoculate. (a) Percent survival. (b) Female fecundity.

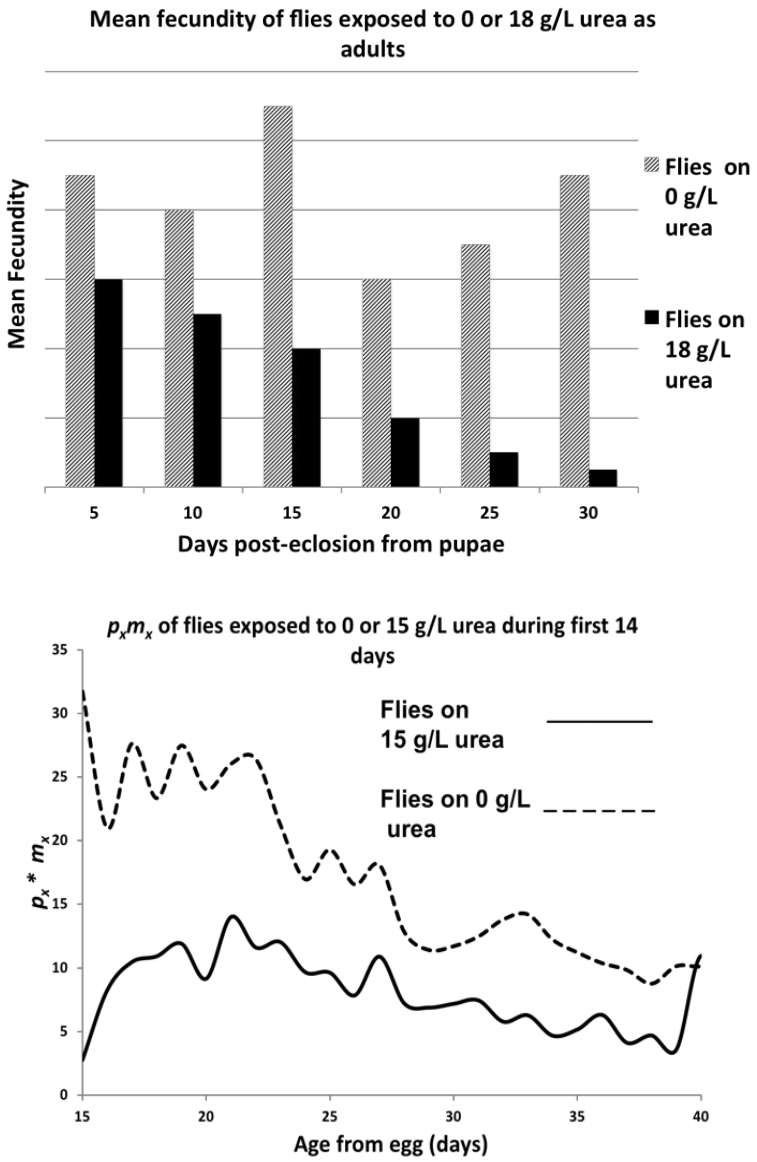

Figure 1.2 The effect of urea on *D. melanogaster*. (a) Data plotted from Joshi et al. (1996) showing mean fecundity of adult flies maintained on 0 or 18 g/L of urea (b) Data from Chapter 2 showing female $p_x m_x$ when exposed to 0 or 15 g/L of urea only in larval stage and first few days of adult stage (prior to day 14). After day 14, flies were given food with no urea.

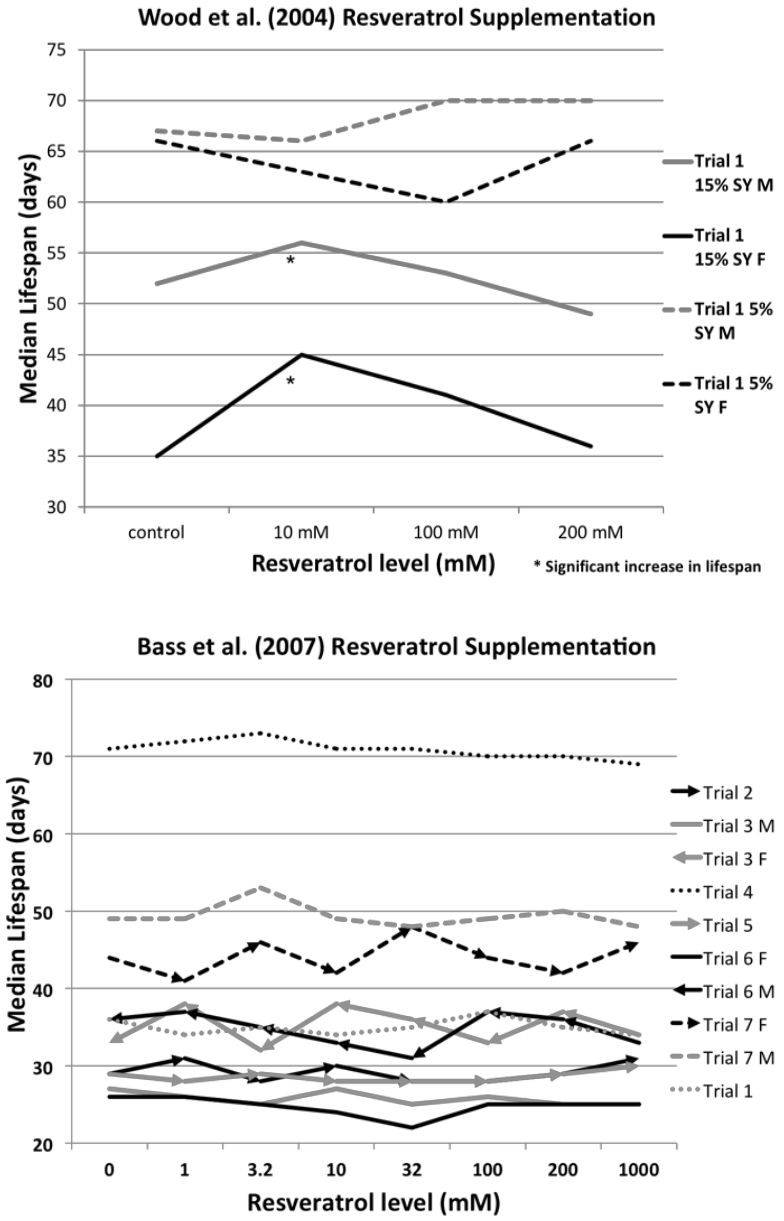

Figure 1.3 Two studies of the dose-dependent effects of resveratrol on the lifespan of *D. melanogaster.* (a) Data from Wood et al. (2004) showing the effect of various levels of resveratrol on median lifespan. Trials using the wild type strain Canton-S under a 15% sugar-yeast diet (solid line) and 5% SY diet (dotted line) are graphed. (b) Data from Bass et al. (2007) showing the effect of various levels of resveratrol on median lifespan. Some trials are split into two trend lines, when males and females were tabulated separately.

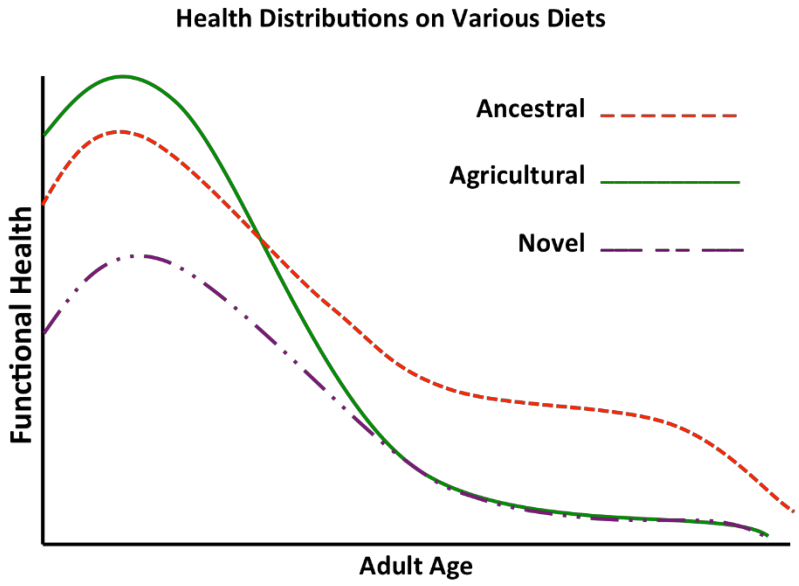

Figure 1.4 The functional health measure in our research with *Drosophila* is usually measured as the product of an individual's probability of survival to a specific adult age and fertility at that age. The graph summarizes recent data of ours in which individuals cope as well or better with an evolutionarily recent "agricultural" diet as on their ancestral diet, but *only* at early ages. At later ages, the Hamiltonian diet hypothesis infers that older individuals should fare better on an ancestral diet, when Hamilton's forces of natural selection have weakened enough to have short-changed adaptation to agricultural food. Individuals raised on evolutionarily novel "industrial" foods fare considerably worse than those raised on ancestral foods at all ages.

TABLES

Table 1.1 Longevity-increasing compounds and their potential adverse effects on healthspan

Longevity stimulating substance	Reduces female fecundity	Possibly sedates	Reduces male mating success	Decreases Viability	Decreases metabolism	Depends on genetic background	Overly dependent on environment	Reference
Urea	X							Joshi et al. (1996)
Lithium		X	X					Matsagas et al. (2009)
resveratrol						X	X	Bass et al. (2007) Wood et al. (2004)
Penylbutyrate (PBA)						X		Kang et al. (2002)
Lamotrigine		X			X			Avanesian et al. (2010)
Deuterium				X				Hammel et al. (2013)

CHAPTER 2

Hamiltonian patterns of age-dependent adaptation to novel diets in *Drosophila melanogaster*

ABSTRACT

A variety of anthropologists and physicians claim that the health of present-day humans would be enhanced by reversion to "Paleo" diets. Against them, a few assert that long-agricultural populations are well-adapted to agricultural diets, due to the speed with which natural selection can fashion effective adaptations to novel diets. But theoretical analysis based on Hamilton's forces of natural selection suggests that both might be incorrect: as the forces of natural selection fall with adult age, we expect adaptation to evolutionarily recent, but not entirely novel, diets to be attenuated with age. Explicit simulations of age-dependent adaptation suggest that populations adapt to a new environment quickly at early ages, but only slowly and incompletely at later adult ages. Experimental tests for age-dependent adaptation to novel diets were performed in populations of *Drosophila melanogaster*. The results provide support for the simulations, with clear trends of age-specificity of adaptation in two laboratory experiments of dietary transition. These findings suggest that humans could revert to an ancestral diet at later ages to alleviate some chronic disorders. However, at earlier ages, long-agricultural human populations might be best able to achieve reasonable health on an organic agricultural diet.

INTRODUCTION

Some evidence suggests that one cause of declining health in modern human populations is the "mismatch" between past human evolution and the diets we are now consuming (Eaton and Konner 1985; O'Keefe and Cordain 2004; Jönsson et al. 2009; Lindeberg 2010). Paleo-enthusiasts argue that our recent dependence on foods derived from grass species (e.g. wheat, corn, rice) and milk exacerbates such chronic disorders as obesity, type two diabetes, and gut disorders such as Crohn's disease (e.g. O'Keefe and Cordain 2004; Eaton and Konner 1985). Some physicians argue that heart disease and dementia are linked to agricultural diets (e.g. Lindeberg et al. 2007; Davis 2011; Perlmutter 2013). Such authors claim that the 10,000 years or so since the beginning of large-scale agriculture and animal domestication was too little time for evolution to adapt our species to the agricultural diet and lifestyle. Therefore, they suggest, humans can best optimize metabolism and physiology when they consume a diet more like that of our ancestors before the advent of agriculture. Moreover, human populations which have recently adopted the agricultural diet exhibit dramatic declines in health (Larsen 1995), which makes it plausible that adopting a Paleolithic hunter-gatherer diet would alleviate many chronic disorders in newly agricultural groups. But it does not demonstrate the validity of the view that all contemporary human populations would similarly benefit from "Paleo" diets and lifestyles, a view that we will refer to here as the "*Paleo hypothesis*."

On the other hand, Zuk (2013) has argued that our evolution has featured enough time to adapt humans to agriculture among those populations with long-agricultural ancestry. Thus, she has proposed that there are no health benefits to be achieved from reverting to Paleolithic diets for most inhabitants of industrialized countries. We will refer to this as the "*anti-Paleo hypothesis*."

Recently, we have developed an evolutionary analysis of the effects of diets from different phases of a species' evolutionary history based on Hamilton's (1966) forces of natural selection. Hamilton's forces of natural selection provide scaling factors for the effect of selection on age-specific components of life history, scaling factors that usually decline with increasing age. Theoretically, selection for adaptation to novel environments is expected to be strongest at younger ages, but then fall toward low intensities during later adult life. This suggests the hypothesis that when adaptation to a novel environment is at least somewhat age-specific, then natural selection will produce more rapid adaptation at early ages compared to later ages, due the weaker forces of natural selection at later ages (Mueller et al. 2011).

A corollary of this general hypothesis for humans specifically is that, when humans with long-agricultural ancestry are young, they are well adapted to agricultural diets and activity patterns. But at later adult ages, with enough age-specificity of the alleles that established our adaptation to agriculture, humans may lose their ability to digest agricultural foods or to cope with the patterns of physical labor characteristic of agricultural life. Thus, our evolutionary hypothesis of age-dependent adaptation suggests that people with long-standing agricultural ancestry might benefit from adopting a Paleolithic diet only at later adult ages. We call this the "*Hamiltonian hypothesis"*. An explicit model for transient age-dependent adaptation supports the intuitive expectation that populations adapt quicker to a novel environment at early ages and slower at later ages. (Figure 2.1; Phung et al. in prep.) This chapter tests the Hamiltonian hypothesis using two models of dietary transition.

First Laboratory Model of Dietary Transition

Since the summer of 1981, the long-standing laboratory *Drosophila* populations used in these experiments have been maintained exclusively on medium that contains banana and high-

sugar syrups as the chief substrates (Rose et al. 2004). The wild *Drosophila* population from which these laboratory populations were derived is that of Northeastern United States; the local agricultural setting is one that has featured apples as the chief cultivated fruit for centuries (Ives, 1970). No banana cultivation occurred over the three centuries or so that wild Drosophila lived in this region, prior to our founding of laboratory populations from them. As a result, we have a model system which features one long-standing dietary regime, dominated by apple rot, being replaced with another long-standing dietary regime, banana with live yeast.

To further study patterns of adaptation to novel environments, we can also impose a novel dietary regime on these flies. The entirely novel dietary regime we use here features oranges as the chief substrate. No orange cultivation occurred in the region inhabited by the wild *Drosophila* we sampled.

If the age-dependent hypothesis is correct, then we expect our lab populations to fare as well or better on banana as on apple at early ages. However, at much later ages, our models predict that these populations should perform better on apple medium, when the forces of natural selection have weakened enough to forestall sufficient adaptation to the banana food provided more recently in their evolutionary history. Furthermore, we expect that flies on banana should outperform flies on an evolutionarily novel diet at early ages. But at later ages, we expect the functioning of flies given entirely novel diets to converge on that of flies fed banana, because a lack of adaptation at later ages to the banana diet.

Moreover, our models predict that the timing of this "switch" to better performance on the apple diet should depend on the life-cycle imposed by the culture regime; that is, it should be dependent on when Hamiltonian forces weaken. In other words, if the reproductive window of our populations is pushed to later ages, our simulations predict a longer period of sustained function

on the banana diet, with the benefits of a switch to the apple diet occurring at later ages or perhaps not at all. We test this hypothesis by subjecting populations of two different reproductive strategies to our various diets. Our qualitative predictions for the patterns of age-specific adaptation to this assortment of diets are shown in Figure 2.2.

Second Laboratory Model of Dietary Transition

In the Fall of 1996, two five-fold replicated stocks, $UX_{1\text{-}5}$ and $AUC_{1\text{-}5}$, were derived in the lab from the $UU_{1\text{-}5}$ (Figure S2.1; Joshi and Mueller 1996). The $UU_{1\text{-}5}$ were derived from the $B_{1\text{-}5}$ (Rose 1984). The UX populations were selected for larval tolerance to toxic levels of urea for approximately 350 generations. The concentration of urea started at 12g/L banana food and was ramped up to 18g/L at generation 20 (Borash et al. 2000). It has since been reduced to 16g/L and has remained at this concentration for hundreds of generations. The AUC populations are maintained identically to the UU populations and serve as the controls to the UX populations. As a result of the derivation of these populations, we have an additional model system which features one long-standing diet, banana without supplemented urea, being replaced by another long-standing diet, urea supplemented banana food.

If the Hamiltonian hypothesis is correct, we expect the urea adapted UX populations to have better performance on the urea diet particularly at early ages compared to the AUC control populations on urea. In addition, the UX populations should fare as well or better on a urea supplemented diet at early ages compared to a standard banana-molasses diet, followed by convergence at later ages. Whether the UX populations perform better on the urea diet compared to the banana diet at early ages will depend on whether adaptations required for healthy function on the urea supplemented food tradeoff with adaptations needed for healthy function on regular banana food. Furthermore, if these tradeoffs are occurring, we predict the AUC control populations

to fare better on the standard banana-molasses diet at early ages compared to the urea adapted UX populations followed by convergence at later ages. Lastly, we would expect the UX populations to have similar performance on the banana diet compared to the AUC populations on the urea supplemented diet only at early ages. At later ages the UX populations should perform better on the banana diet compared to the AUC populations on the urea supplemented diet.

MATERIALS AND METHODS

Populations: We used large, outbred populations of *Drosophila melanogaster* selected for three different patterns of age-specific reproduction. Five "ACO" replicates have been adapted to banana-molasses food for ~1000 generations and have a 10-day life cycle. Five "CO" replicates have been adapted to banana-molasses food for ~500 generations and have a 28-day life cycle. The AUC and UX populations have a 21-day life-cycle (Figure S2.2). A more detailed and up-to-date description of the history and culture methods for the ACO and CO populations can be found in Burke et al. (2016). A more detailed description of the AUC and UX populations can be found in Borash et al. (2000).

Overall experimental design: A total of four diet-manipulation experiments were performed in the lab. ACO replicates 1-3 were assayed on the banana and orange diets. ACO replicates 1-5 were assayed on the apple and banana diets. CO replicates 1-5 were assayed on the banana and apple diets. The AUC and UX populations were assayed on the banana and urea supplemented banana diets.

Food Preparation: The evolutionarily recent banana-molasses food given to fly cohorts is composed of the following ingredients per 1L distilled H_20: 13.5g Apex® Drosophila agar type II, 121g peeled, ripe banana, 10.8mL light Karo® corn syrup, 10.8mL dark Karo® corn syrup, 16.1mL Eden®organic barley malt syrup, 32.3g Red Star® active dry yeast, 2.1g Sigma-Aldrich® Methyl 4-hydroxybenzoate (anti-fungal), and 42.5 mL EtOH. The novel orange food was prepared identically to the banana-molasses food except peeled banana was replaced with juice and pulp from peeled oranges. The long-ancestral apple food is prepared in the same manner as the banana food, except the diet lacks the barley malt and corn syrups, and we substitute organic peeled applesauce for the peeled banana. This is our best emulation of the ancestral diet of our lab flies.

Basic nutritional facets of each diet are shown in Table S2.1. Our experimental cohorts are exposed to each of these foods throughout both their developmental stages and adulthood. The urea diet used in our experiments with the UX and AUC populations is prepared in the same manner as the standard banana-molasses diet except that 16g of urea is added per liter of banana food. Urea is added to the banana food and then blended to ensure that the urea is evenly dispersed in the banana food. Flies are only exposed to the urea during their developmental phase in vials. Flies in the urea diet experiments are given regular banana food as adults.

Mortality and fecundity assays: All populations were reared in polystyrene vials with the respective diet and given 9 (ACO) and 14 (CO/AUC/UX) days to develop. Adult flies were transferred into cages on the 9^{th} (ACO) and 14^{th} (CO/AUC/UX) day using CO_2 anesthesia and given fresh food every day with 1mL supplemented yeast (98mL distilled water, 2g active dry yeast, and 2mL 1% acetic acid). Individual mortality was assessed every 24 hours, the flies were sexed at death, and the observed cohort size was calculated from the complete recorded deaths. During the assay, flies were transferred to clean cages once a week using CO_2 anesthesia. Cohorts were assayed in 6L cages at ~1000 flies per cage. Flies were transferred to 3L cages at 50% starting cohort size to control for density effects. Age-specific fecundity was also assessed every 24-hours, being estimated from the number of eggs laid by females on the culture medium plates placed in each mortality assay cage, divided by the number of females still alive. Media plates were washed on filter paper with the lab's fecundity funnel system and then scanned for counting at a later time. Egg counting was performed using ImageJ, a National Institute of Health image-processing program.

$p_x m_x$ analysis for ACO experiments: The age-specific survival probability *(p_x)* is the probability of a female surviving to age *x,* given that she survived to the start of the age-interval. It is calculated using the following equation:

$$p_x = 1 - \left(\frac{d_x}{n_x}\right)$$

where d_x is the number of females that die at age *x,* and n_x is the number of females that were alive at the start of age *x*. Age-specific fecundity (m_x) is the average number of eggs laid per surviving female at age *x*. The product of these two variables gives an overall estimate of how cohorts of females are functioning at each age. In our experiments, the unit interval for *x* is a single day.

For all three diets, $p_x m_x$ remained roughly steady until a "break-day" when we see a linear decline in this parameter until day 39 (Figure S2.3). The model we fit to this data was a three-parameter two-stage linear model with the following relationship between age (x) and $p_x m_x$:

$$p_x m_x = \begin{cases} a_0, if\ x \leq\ a_2 \\ a_0 + a_1(x - a_2), if\ x > a_2 \end{cases}$$

The model was fitted using all the $p_x m_x$ data at each age starting at the first day of the assay (day 10 from egg). This model was fit to the data using a nonlinear least-squares function in the R-project for statistical computing (https://www.r-project.org). We wrote a self-starting R-function for the two-stage linear model that provided initial estimates for the parameter values as well as the predicted $p_x m_x$ from the equation above (4). A significance value of 0.05 (α) was used to test the null hypothesis that the slopes or y-intercepts of the two linear regressions for each diet for each regression analysis were not different.

$p_x m_x$ analysis for CO experiment: In this experiment we tested for differences in $p_x m_x$ in seven, five-day age-classes in CO populations exposed to the apple and banana diets. The observations consisted of $p_x m_x$ at an age (x) within an age interval (k = 1, 2,…,7). Within each

interval, p_xm_x was modeled by a straight line allowing diet (j=1 for banana, or j=2 for apple) to affect the intercept, but not the slope of the line. Slope could vary between intervals. Populations (i = 1, 2…,10) contributed random variation to these measures. With the notation above, the p_xm_x at age (x), interval (k), diet (j), and population (i) is y_{ijkx} and can be described by,

$$y_{ijkx} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)x + \delta_k\delta_j\mu_{jk} + c_i + \mathcal{E}_{ijkx},$$

where $\delta_s = 0$ if s = 1 and 1 otherwise, and c_i and $\mathcal{E}_{ijkx}$ are independent standard normal random variables with variance σ_c^2 and $\sigma_\mathcal{E}^2$, respectively. The effects of diet on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}. Statistical computing was completed in R (https://www.r-project.org).

p_x survivorship analysis: For each combination of *treatment*sex*, three-day survivorship intervals were computed in the R-project for statistical computing (https://www.r-project.org). For each interval a new categorical variable was then created, defining the status of each one of the flies (0 = dead or 1 = alive). The counts of each interval were used in a chi-squared test to compare treatments (ACO banana vs. orange, ACO apple vs. banana, or CO apple vs. banana). A Bonferroni correction was applied to correct for the multiple age-classes per comparison. A p-value of less than 0.05 was considered statistically significant. Each of the six comparisons for the AUC and UX populations were analyzed in the same manner.

p_xm_x analysis for AUC and UX experiment: Differences in p_xm_x were analyzed using a student's t-test in the R-project for statistical computing (https://www.r-project.org). Two-day average p_xm_x was calculated per replicate and the following comparisons were test with a student's t-test (AUC banana vs AUC urea, AUC Banana vs UX urea, UX banana vs. UX urea, AUC urea vs. UX urea, UX banana vs. AUC urea, AUC banana vs. UX banana). A Benjamini-Hochberg procedure was used to correct for the multiple tests completed over adult age.

Mean Longevity analysis: Mean longevity was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The model used for the data is described as follows: Let y_{ijkm} be the longevity for diet – i (i =1 (ACO banana), 2 (ACO apple)), sex-j (j=1 (female), 2(male)), population – k (k=1,.., 10) and individual – m (m=1,.., n_{jk}). A LME model for longevity is,

$$y_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where $\delta_s = 0$, if s = 1, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively.

RESULTS

ACO apple and banana diet results: For $p_x m_x$, the *y*-intercepts of the first stage regression (a_0) for each diet were not statistically different (p=0.763, Table 2.1). Flies performed roughly similarly on the banana and apple diets at ages up to the break-day (~26 days from egg) (Figure 2.3). The break-days (a_2) for each diet were also not statistically different (p=0.775, Table 2.1). When observing the second stage slope (a_1), we see that that the slope for the banana diet is significantly more negative than that for the apple diet (p=0.00920, Table 2.1). $p_x m_x$ declined faster on the banana diet after the break-day, relative to life history on the apple diet (Figure 2.3).

We did not observe a significant difference in mean longevity between these two diets (Table S2.2). In addition, we see several significant intervals where age-specific survivorship (p_x) is higher on the banana diet compared to the apple diet (Figure S2.4). Particularly, male and female flies recently eclosed from pupae (day 10-12 from egg), show higher mortality rates on the apple diet, suggesting that performance is lower on the apple diet compared to the banana diet at juvenile ages prior to adult (Figure S2.4). The survivorship and mortality curves for these diets are shown in Figure S2.5 and S2.6 respectively.

Recall that our prediction is a less rapid decline in $p_x m_x$ at later ages among flies given our crude evocation of their ancestral diet, compared to the decline expected with their recent banana diet. We found that the regression of $p_x m_x$ on age with the apple diet to be statistically less negative at later ages, compared to the regression of $p_x m_x$ on age with the banana diet. We observed no statistical difference for $p_x m_x$ between the two diets at earlier ages.

ACO orange and banana diet results: For $p_x m_x$, the *y*-intercepts of the first stage regressions (a_0) for each diet were statistically different (p=0.043, Table 2.2). Flies performed better on the banana diet compared to the orange diet at ages up to the break-day (~28 days from

egg, Figure 2.4). The break days (a_2) for each diet were also not statistically different (p=0.123, Table 2.2). When observing the second stage slope (a_1), we see that that the slope for the banana diet and the orange diet are not significantly different (p=0.922, Table 2.2). $p_x m_x$ declined at a similar rate on the banana and orange diet after the break-day (Figure 2.4).

Male flies on the orange diet have a significantly lower mean longevity compared to the banana flies, however this significant difference was not observed in females (Table S2.2). Results from the survivorship analysis for the banana and orange diets are shown in Figure S2.7. The survivorship and mortality curves for these diets are shown in Figure S2.8 and S2.9 respectively.

Our prediction was a lower regression for the orange diet at earlier ages compared to the banana diet. We found that the regression of $p_x m_x$ on age with the orange diet to be statistically lower at ages up to the break-day, compared to the regression of $p_x m_x$ on age with the banana diet. We observed no statistical difference for $p_x m_x$ between the two diets at later ages. Performance after the break-day was similar between the two diets.

CO apple and banana results: For the first adult-age interval (days 15-19 from egg), we did not observe a significant difference between the two diets (p=0.0633, Table 2.3, Figure 2.5). For the following two intervals (days 20-24 & 25-29 from egg), we observed significantly higher $p_x m_x$ intercepts on the banana diet compared to the apple diet (p<0.05, Table 2.3, Figure 2.5), followed by convergence in the last four intervals (p>0.05, Table 2.3, Figure 2.5). For the last age-class, we see a switch to higher performance on the apple diet; however, this was not statistically significant. Mean longevity and survivorship is significantly higher in the CO banana compared to the CO Apple. (Figures S2.10-2.12; Table S2.2)

The results of this last experiment fit the prediction of sustained CO function on banana further into adult age, compared to the ACO flies. Furthermore, we obtained the expected convergence in $p_x m_x$ trends at later age classes. We observed higher $p_x m_x$ on the apple diet for the last interval, but this difference was not significant.

AUC and UX banana and urea results: $p_x m_x$ results for the six different comparisons are shown in Figure 2.6. AUC banana has significantly higher $p_x m_x$ compared to the AUC urea for every interval except for the last (Figure 2.6a). We see significantly higher $p_x m_x$ in the AUC banana compared to the UX urea for the first three age classes, followed by convergence at later ages (Figure 2.6b). Interestingly, we see a trend towards convergence by the reproductive window in this comparison with no difference in $p_x m_x$ seen for day 21-22 from egg (Figure 2.6b). For the UX banana and UX urea comparison, we see significantly higher $p_x m_x$ in the banana treatment at the first interval of the assay followed by a switch to higher $p_x m_x$ on urea for days 17-22 from egg. After this age we see no difference in $p_x m_x$ for the following age-classes (Figure 2.6c). The UX urea statistically outperforms the AUC urea for most intervals (Figure 2.6d). UX banana has a significantly higher $p_x m_x$ for the first interval followed by quick convergence for days 17-22 from egg. The UX banana treatment then significantly outperforms the AUC urea treatment for the remaining age classes (Figure 2.6e). Lastly, the AUC banana treatment has higher $p_x m_x$ for early ages prior to the populations' reproductive window followed by overall convergence in $p_x m_x$ after day 22 from egg (Figure 2.6f).

DISCUSSION

The findings displayed in Figures 2.3-2.6 were obtained from experiments that studied *Drosophila* populations that had an inadvertent dietary transition during their evolution. In other words, we did not directly monitor forward selection to a novel diet. However, by performing diet manipulation experiments on such populations with well-characterized dietary histories, we still can test whether populations are conforming to the Hamiltonian hypothesis of age-dependent adaptation, rather than the patterns expected from both Paleo and anti-Paleo hypotheses which do not consider age-specificity.

The results of the diet manipulation experiments using the ACO populations indicate that adaptation appears to conform to Hamiltonian predictions: younger adult flies fare well on their evolutionarily recent banana diet, while older adult flies fare better on medium that is relatively more like their evolutionarily ancestral apple diet. Notably, this occurs despite their lack of exposure to this ancestral diet for more than 1,000 generations of laboratory evolution. Survivorship analysis in these populations indicates that there may be a decrease in functional health at early ages on the apple diet compared to the banana diet (Figure S2.4, S2.6), however the $p_x m_x$ analysis performed did not detect a difference. The results from the CO experiment provide more evidence for our hypothesis. These late-cultured flies performed significantly better on the banana diet at ages prior to the later reproductive window and the fall in Hamilton's forces. Flies performed better on the apple diet in the last interval, though this difference was not statistically significant.

The results from the AUC and UX populations provide further support for the Hamiltonian hypothesis. The gain and loss of adaptation in these populations is clearly strongest at earlier ages prior to their reproductive windows, when Hamilton's forces of natural selection are at their

greatest. Notably, at early ages, the UX flies fed urea food outperform UX flies fed standard banana food, while we see no differences between these treatments at later ages. In addition, there is a clear trend toward convergence in $p_x m_x$ in the UX banana and AUC urea feed flies at early ages, however at later ages the UX populations exposed to banana food outperform the AUC flies exposed to the urea. Taken together, it is clear that there is an age-dependent loss of adaptation on the banana food and gain of adaptation on the urea food in the UX populations. Further support for this statement is provided by results from the AUC banana and UX banana comparison. The AUC banana flies generally outperform the UX banana flies at early ages while we see no differences at later ages.

In these experiments we have shown that age-specific adaptation to a novel environment proceeds at different rates for early-life phenotypes vs. late-life phenotypes, in large-scale experiments. The ages at which we might expect to see a rapid transition from a maladaptive phenotype to a well-adapted phenotype depend on several factors: the time since moving to the new environment; the severity of selection acting on new genetic variants; the magnitude of the phenotypic effects among new mutants; and the effective population size. Additional theoretical and experimental work on such issues is an obvious next step for this research.

Medical Application

If our basic findings are broadly applicable, they are relevant to ongoing discussions of optimal diet choices for present-day human populations. Anthropologists and physicians have focused on the high incidence of age-related disorders (e.g. type II diabetes and cardiovascular disease) in populations that have transitioned to the agricultural diet within recent human history (e.g. Lindeberg 2010; Broadhurst 1997; Carter et al. 1996; Larsen 1995), populations that have not been selected for successful reproduction under agricultural conditions for more than a few

generations. Some of these authors claim that reversion to the diet we had prior to the introduction of grain and dairy foods to our diet, sometimes called the *Paleolithic* or *hunter-gatherer diet*, might help prevent these age-related diseases, especially cardiovascular conditions (e.g. Jönsson et al. 2009). Their clinical data suggests that hunter-gatherer populations with little recent history of agriculture are indeed harmed by the agricultural diet, because those populations are not well adapted to the agricultural diet (e.g. Lindeberg et al. 2007).

They further contend that the introduction of agriculture and animal domestication over the last 10,000 years has left very little time for long-agricultural populations to adapt their essential metabolic and physiological processes to this major change of diet (Klonoff 2009). Note that this additional conclusion is premised on hypotheses concerning the speed of evolution in Mendelian populations, as well as an absence of age-dependence in such evolution. Specifically, they evidently think of the evolution of adaptation in response to natural selection as rather slow, much as Darwin did. Zuk's (2013) argument against the Paleo hypothesis is based on a reading of the available evidence about speed of evolution. Her conclusion is that this assumption of negligible adaptation to the agricultural diet is not correct. Therefore, the validity of standard Paleo theorizing depends on an assumption about the rate of evolution when natural selection is strong that is no longer generally accepted by evolutionary biologists who study the impact of episodes of strong selection (e.g. Grant and Grant 2008).

Our results circumscribe the arguments on both sides of the diet question. The findings presented here suggest that young people from populations with long histories of agriculture may be well adapted to agricultural diets which resemble, to at least some extent, those diets that their ancestors consumed over the last five to ten thousand years. But at later ages, even in such populations, the present results suggest that such adaptation to agricultural life may fail, possibly

to a limited extent during middle age, but perhaps to a catastrophic degree much later in adult life. Laboratory and clinical studies testing the possibility of an age-differentiated impact of altered diets will determine the validity of these theoretical results to the human case, although there is already age-specific data concerning human adaptation to milk consumption as a function of specific populations' histories of animal husbandry. For example, Lindeberg (2010) provides data on maintenance of lactase function with age as well as the incidence of chronic diabetes on present-day diets as a function of agricultural history (see Fig. 4.20 in Lindeberg 2010).

In the meantime, since the present theoretical analysis is general to any population that has undergone a recent dietary transition, there is the possibility of testing the Hamiltonian hypothesis further on model systems in which there has been a change in diet like that of our *Drosophila* populations.

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FIGURES

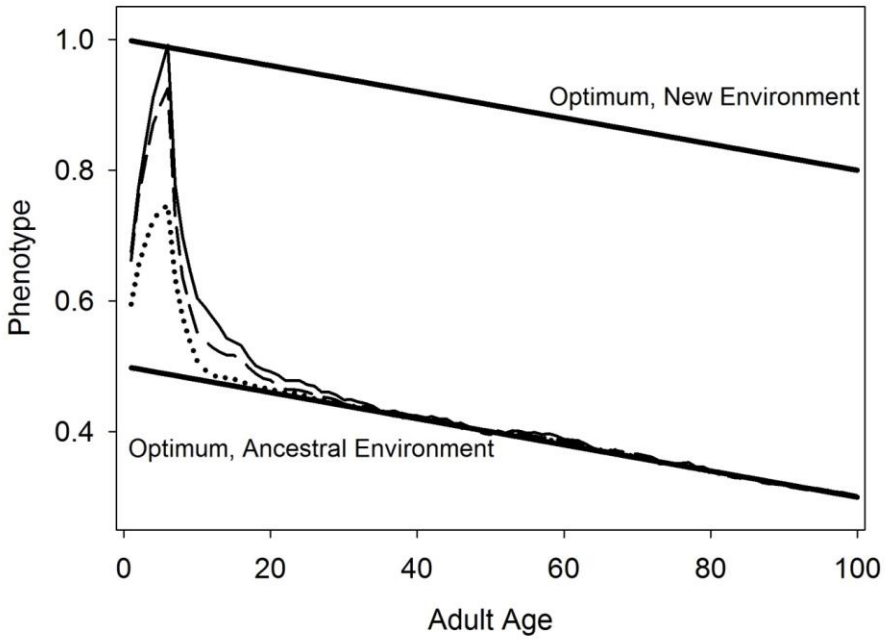


Figure 2.1 An explicit model of transient age-dependent adaptation. The straight lines at the top and bottom show the optimum phenotype for new and the ancestral environments, respectively. When an evolving population shifts to a new environment in which the optimum age-specific phenotype is changed, natural selection will tend to move the population towards that new optimum at each age. But it is much faster at this early in adult life compared with later in adult life. The curves in between the two optimum lines show the intermediate steps of the evolving population's phenotype, with the dotted line giving the initial evolutionary response, the dashed line showing a later intermediate step, and the thin solid line showing the evolutionary outcome still later in the process of adaptation (figure from Rose et al. 2014).

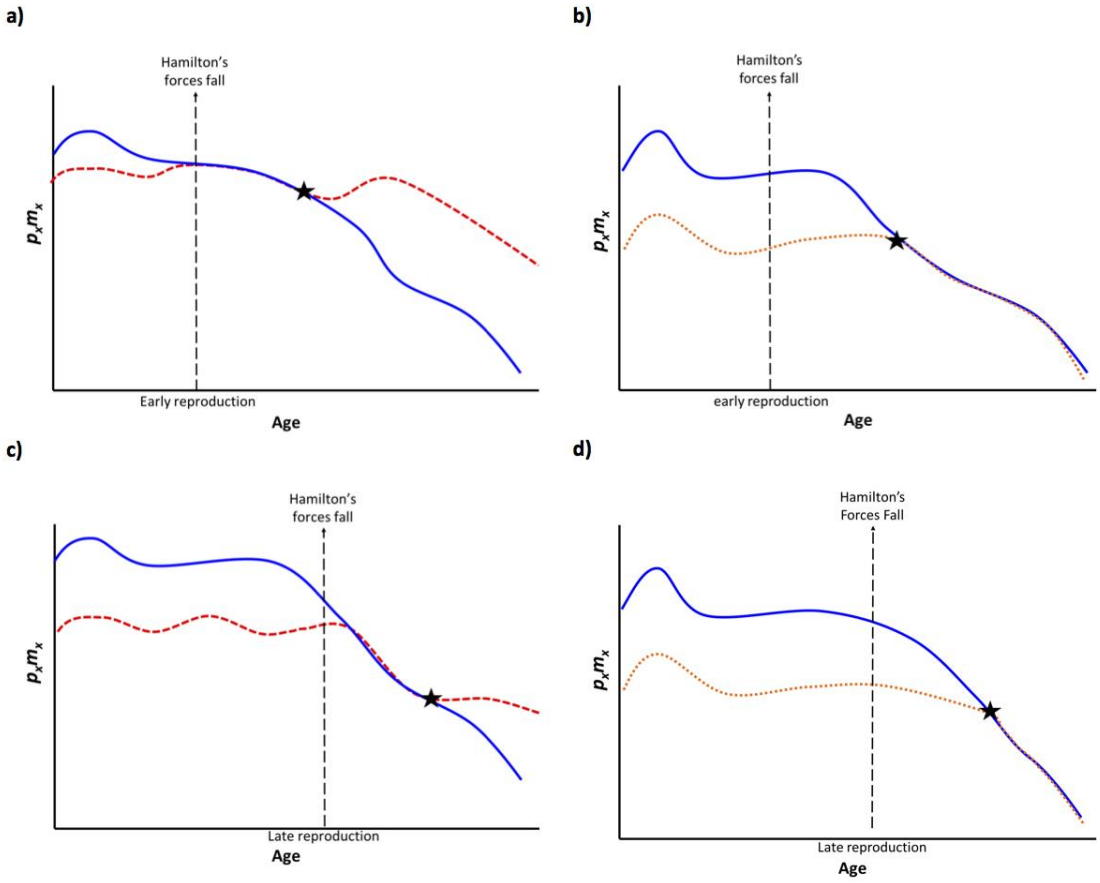


Figure 2.2 Hypothetical $p_x m_x$ curves of flies from two different demographics exposed to various diets. $p_x m_x$ is age-specific survival probability multiplied by age-specific female fecundity. Dashed red trend lines represent $p_x m_x$ on a long ancestral diet, solid blue on an evolutionarily recent diet, and dotted orange on an entirely novel diet. (a) Flies evolving under an early reproduction regime should fare as well or better on their evolutionarily recent diet as on a past ancestral diet at early ages. But at later ages, our simulations predict that individuals should fare better on a long ancestral diet, when the forces of natural selection have weakened enough to prevent sufficient adaptation to the food imposed in recent evolutionary time. (b) Flies should perform significantly better on an evolutionarily recent diet as opposed to an entirely novel diet, particularly at early adult ages. (c) If reproduction is pushed to later ages, we would expect a longer period of sustained function on the evolutionarily recent diet and a switch to superior function with the ancestral diet to occur at later ages or not at all. (d) Our theory also implies a longer period of sustained function on the recent diet compared to an entirely novel diet. Convergence in these trends would be expected at even later ages. This last prediction was not tested in our experiments.

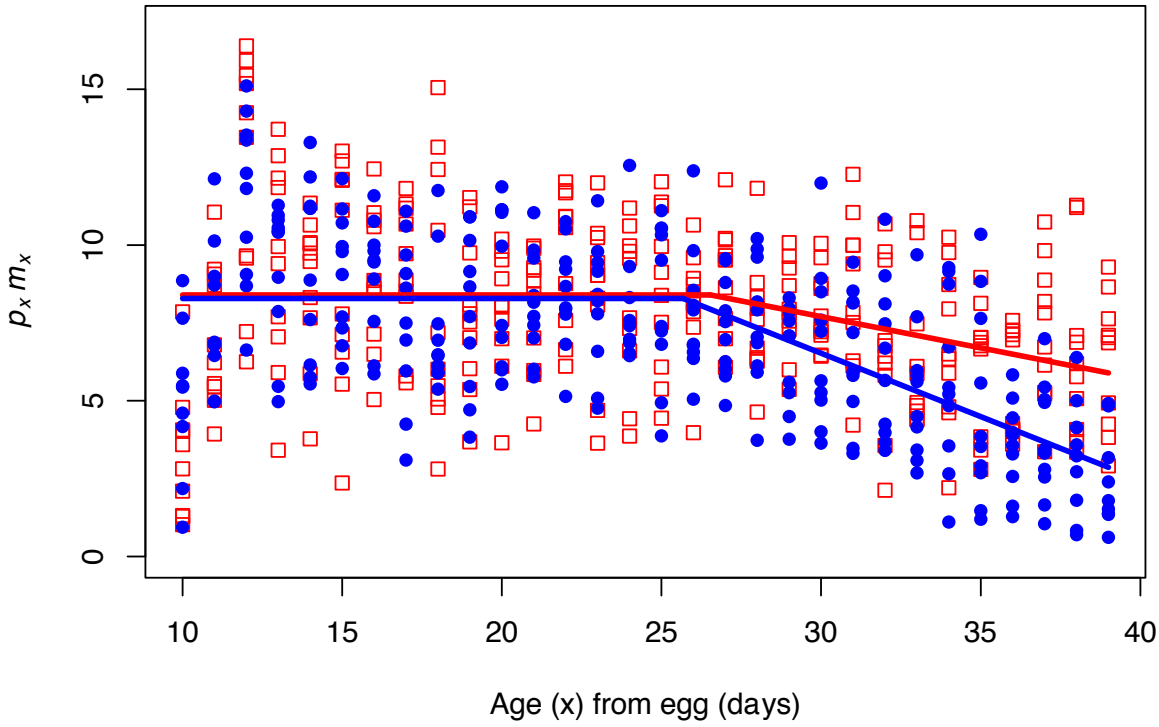


Figure 2.3 $p_x m_x$ results across two experiments with the five ACO populations exposed to our apple and banana diets. Closed blue circles and solid blue lines represent data and regressions from flies fed the banana diet. Open red squares and dotted red lines represent data and regressions from flies fed the apple diet. A significant difference between diets in the intercept from the first stage (a_0) was not observed. In addition, there was not a significance difference in the "breakday" (a_2) between diets. Lastly, the second-stage slope (a_1) was significantly more negative for the banana diet.

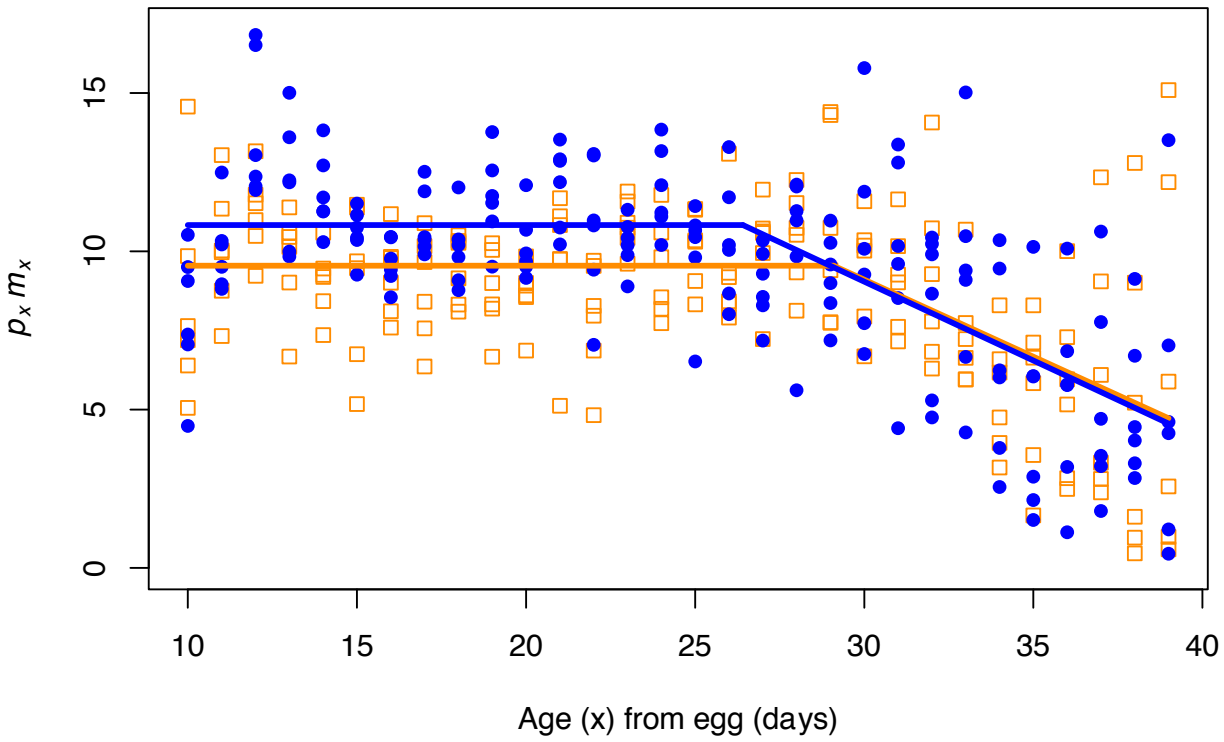


Figure 2.4 $p_x m_x$ results in the three ACO populations exposed to our banana and orange diets. Closed blue circles and solid blue lines represent data and regressions from the banana diet. Open orange squares and dotted orange lines represent data and regressions from the orange diet. A significant difference between diets in the intercept from the first stage (a_0) was observed with flies on the orange diet not performing as well compared to their performance on the banana diet. In addition, there was not a significance difference in the "breakday" (a_2) between diets. Lastly, the second-stage slope (a_1) was not significantly different between the two diets.

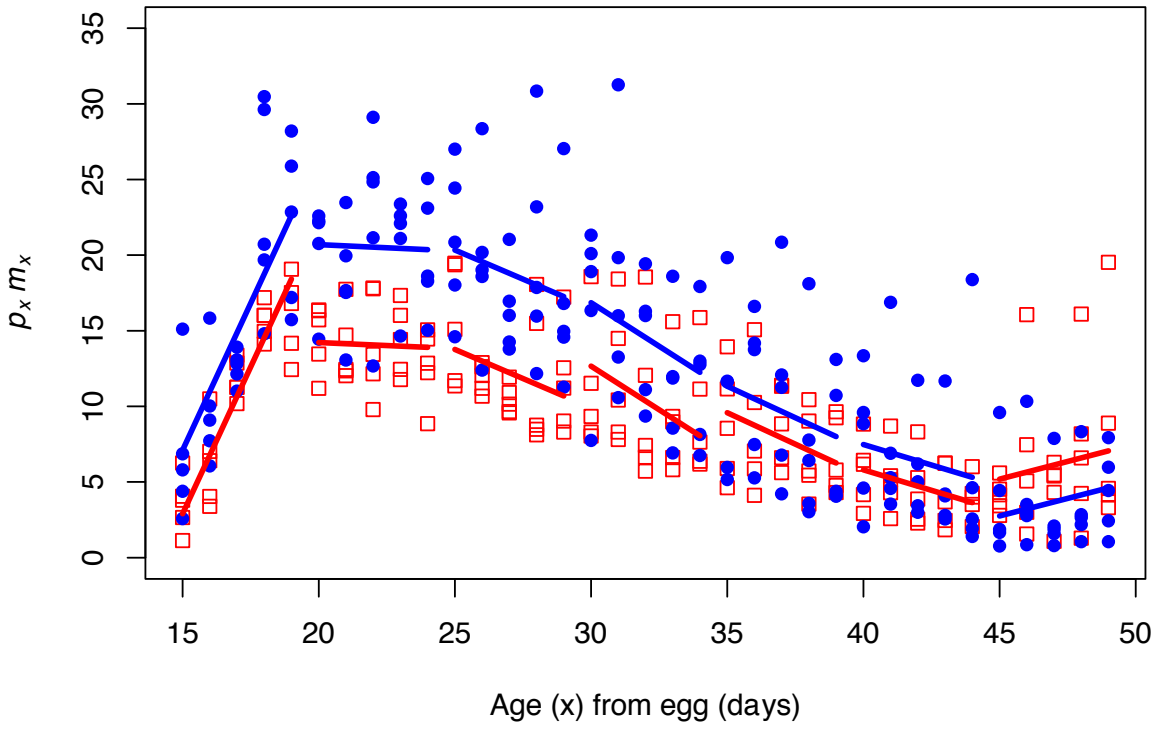


Figure 2.5 $p_x m_x$ results in five CO populations exposed to our apple and banana diets. Closed blue circles and solid blue lines represent data and regressions from the banana diet. Open red squares and dotted red lines represent data and regressions from the apple diet. Flies performed significantly better on the banana diet for the second and third age classes. All other age classes are not significantly different with respect to their performance on the two diets.

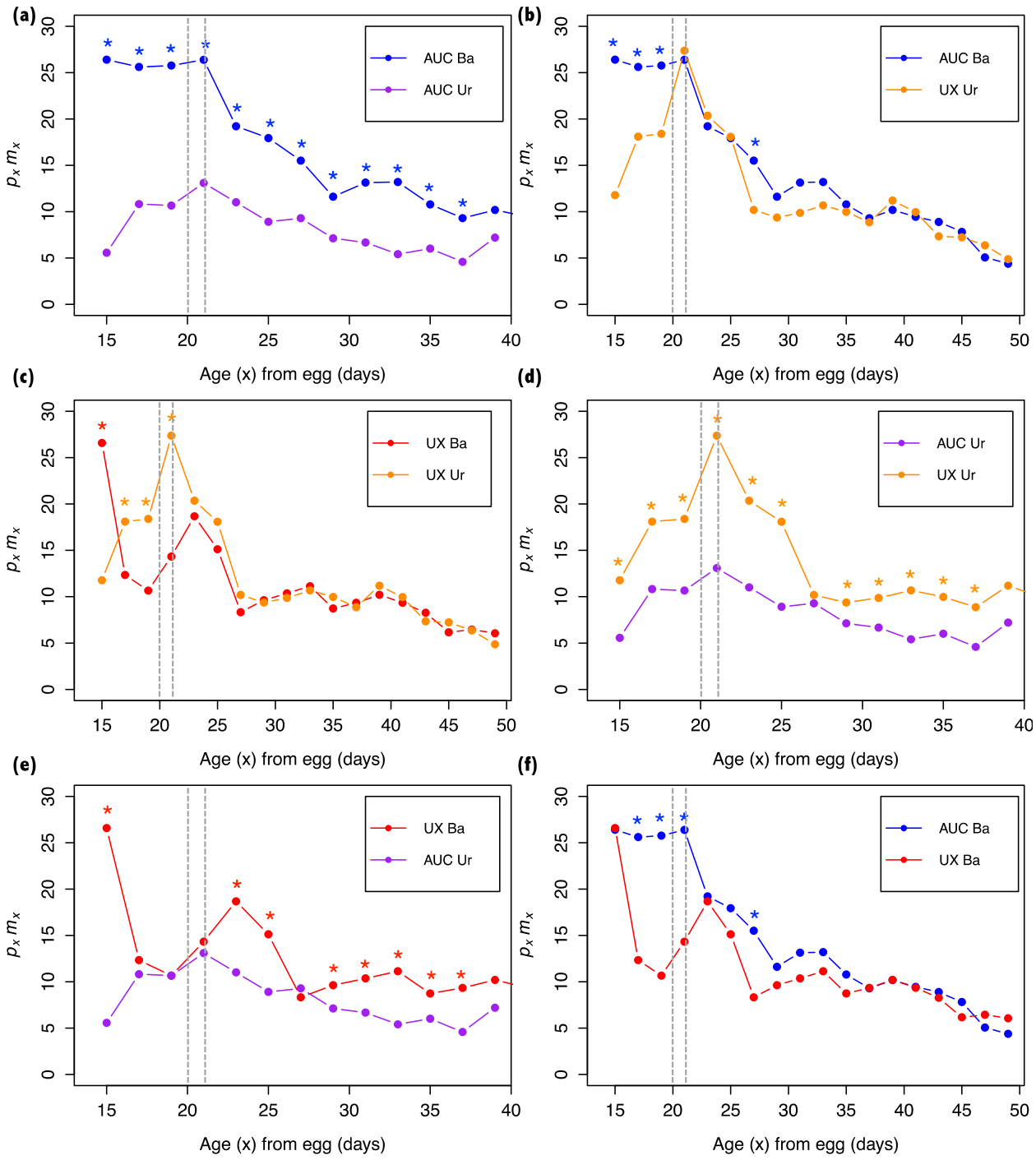


Figure 2.6 $p_x m_x$ results in the AUC and UX banana and urea experiments. (a) AUC Ba vs. AUC Ur (b) AUC Ba vs. UX Ur (c) UX Ba vs. UX Ur (d) UX Ur vs. AUC Ur (e) UX Ba vs. AUC Ur (f) AUC Ba vs. UX Ba. Points represent average $p_x m_x$ across five replicates averaged across two days. *denotes significance for that interval between shown diets ($p<0.05$). The color of the asterisk signifies which treatment has higher survivorship for that interval. Grey lines denote the reproductive windows in these populations (day 20-21 from egg). Ba = banana food, Ur = urea supplemented food

TABLES

Table 2.1 Parameter estimates from the two-stage linear model fitted to $p_x m_x$ data from the ACO's treated with the banana and apple diets (p-values less than 0.05 are bolded).

	First stage *y*-int (a_0)	Second stage Slope (a_1)	"break-day" (a_2)
apple	8.398	-0.201	26.51
banana	8.283	-0.407	25.68
p-value	0.763	**0.0092**	0.775

Table 2.2 Parameter estimates from the two-stage linear model fitted to $p_x m_x$ data from the ACO's treated with the banana and orange diets. (p-values less than 0.05 are bolded).

	First stage *y*-int (a_0)	Second stage Slope (a_1)	"break-day" (a_2)
orange	9.543	-0.486	29.091
banana	10.826	-0.498	26.412
p-value	**0.043**	0.922	0.123

Table 2.3 Estimates of $p_x m_x$ intercepts for each age class (days from egg) in the CO's on the banana and apple diets and p-value of their statistical comparison.

Age-class	Age	$p_x m_x$ apple-banana	*p*-value
15-19	3.88	-4.20	0.0633
20-24	-0.08	-6.90	**0.0106**
25-29	-0.77	-6.58	**0.0098**
30-34	-1.15	-4.19	0.0638
35-39	-0.83	-1.75	0.394
40-44	-0.54	-1.68	0.4152
45-49	0.47	2.45	0.2455

$p_x m_x$ estimates represents the Y-intercepts of a linear regression through all replicates of five-day intervals. *p*-values less than 0.05 are bolded.

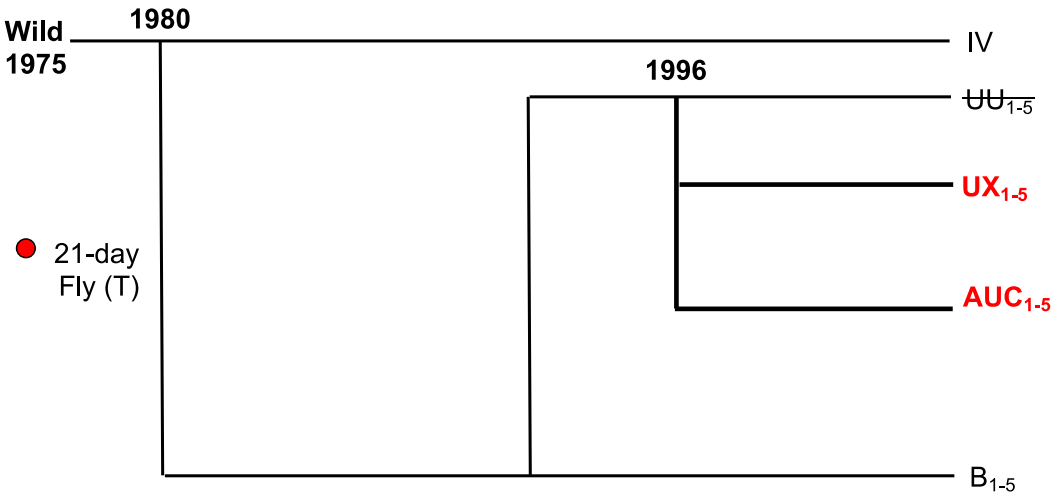
Supplementary Materials

Table S2.1 Nutritional facts for three different diets used in the diet manipulation experiments.

	Banana (1L)	Orange (1L)	Apple (1L)
Total Fat	1.2 g	0.9g	0.7g
Sugar	37.2 g	32.2g	11.1g
Total Carbs	90.36g	76.4g	26.5g
Protein	21.2g	20.9g	19.7g
Calories	450 Kcal	395.6 Kcal	197.66 Kcal
Calculations from the fruit, yeast, and syrups			

Table S2.2 Mean longevity results for experiments using the CO and ACO populations. *p*-values less than 0.05 are bolded. se = standard error for the difference.

Comparison	Male			Female		
	Difference (days from egg)	se	*p*-value	Difference (days from egg)	se	*p*-value
ACO Banana - Apple	1.64	0.80	0.06	0.95	0.80	0.25
ACO Banana - Orange	1.89	0.62	**0.01**	0.23	0.62	0.72
CO Banana - Apple	5.53	1.2	**0.009**	5.05	1.24	**0.02**



	Egg → Day 14	Day 14 → Day 21
AUC_{1-5}	Banana	Banana
UX_{1-5}	Urea	Banana

Figure S2.1 Derivation of the urea-tolerant (UX) and unselected controls (AUC). These three selection regimes are five-fold replicated and maintained at large population sizes (~1000). The UX and AUC populations were derived from the five UU populations (now extinct) (Joshi and Mueller 1996). The UU populations were derived from the B populations (Rose 1984). The AUC and UX populations are maintained on a three-week life-cycle.

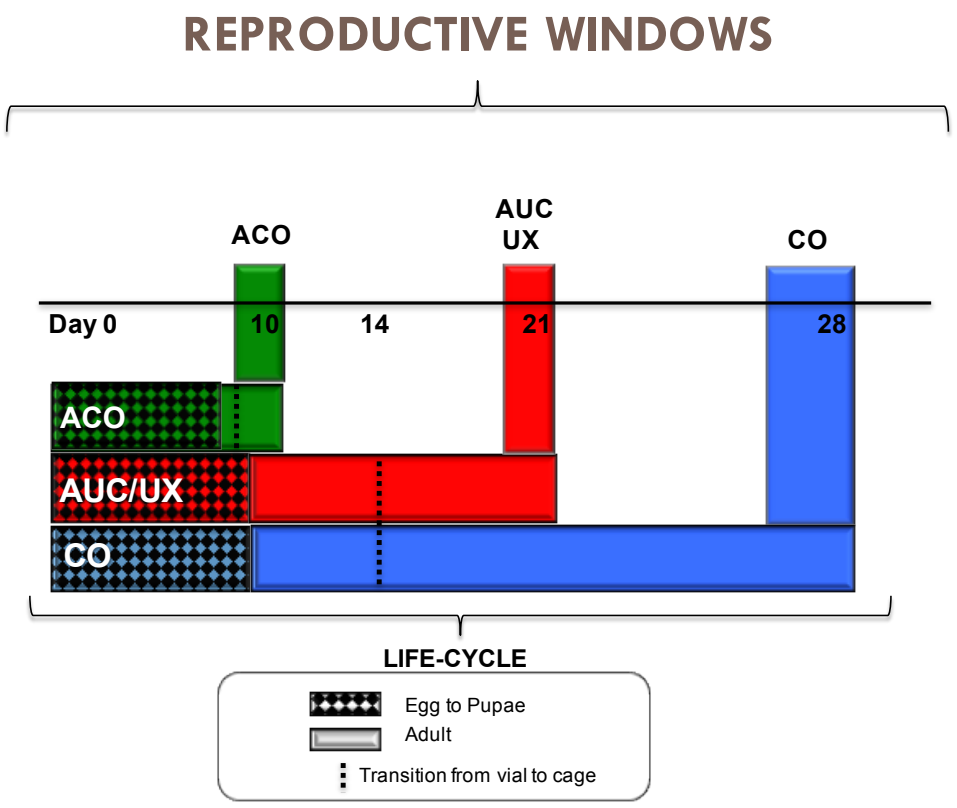


Figure S2.2 Schematic of the life-cycle of the populations used in our experiments. ACO and CO populations are reared in vials for the first 9 and 14 days, respectively. The AUC and UX populations are reared in vials for 14 days. On the 9th and 14th day, adults are transferred to cages and maintained until their respective reproductive windows. Eggs laid during the reproductive window are used to start the next generation.

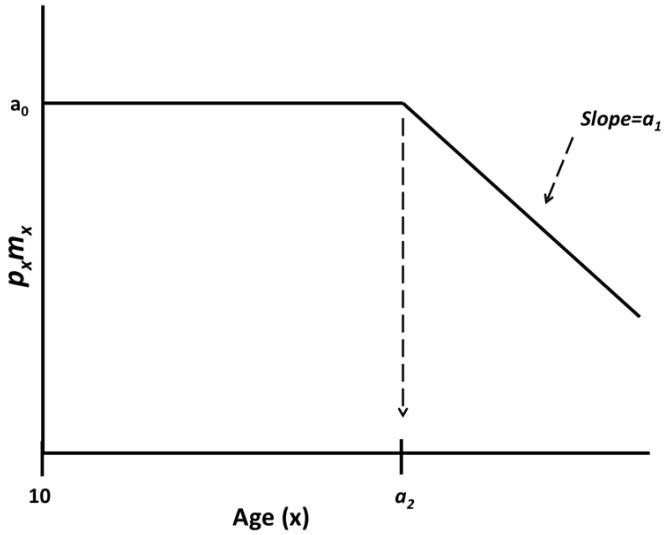


Figure S2.3 The model used to fit the ACO data from our diet manipulation experiments. From the start of the assay (day 10) until the "breakday" (a_2), p_xm_x at age x is described by the horizonal line $p_xm_x = a_0$. After a_2, p_xm_x begins to decline and is described by the line $p_xm_x = a_0+a_1(x-a_2)$.

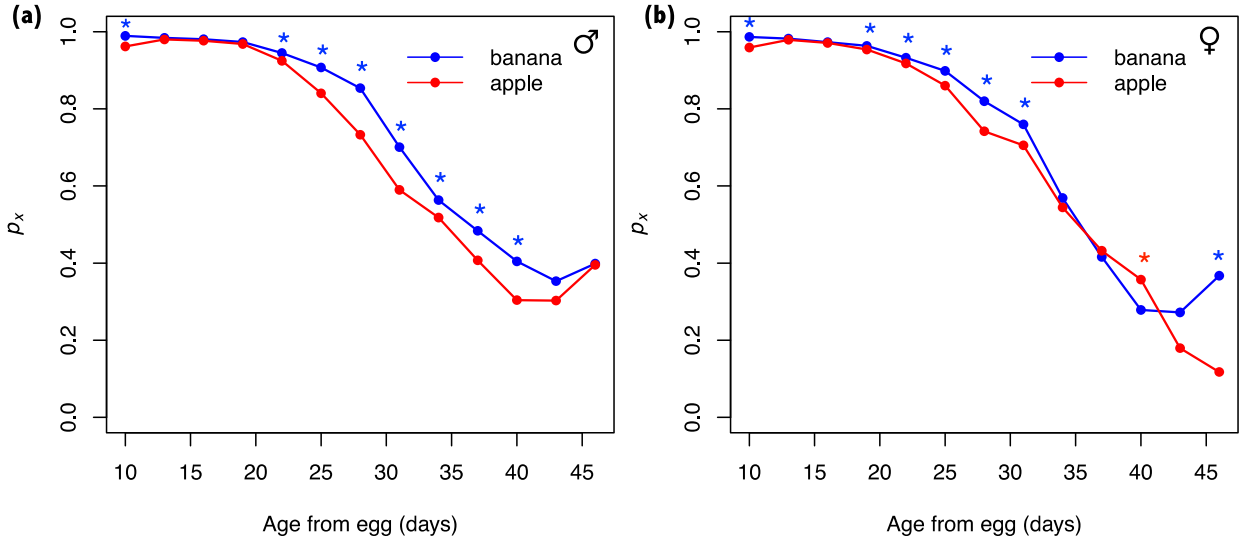


Figure S2.4 ACO conditional survival probability (p_x) over adult age from egg (x) for the apple vs. banana diet. (a) males and (b) females. Points represent p_x pooled across replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk signifies which treatment has higher survivorship for that interval.

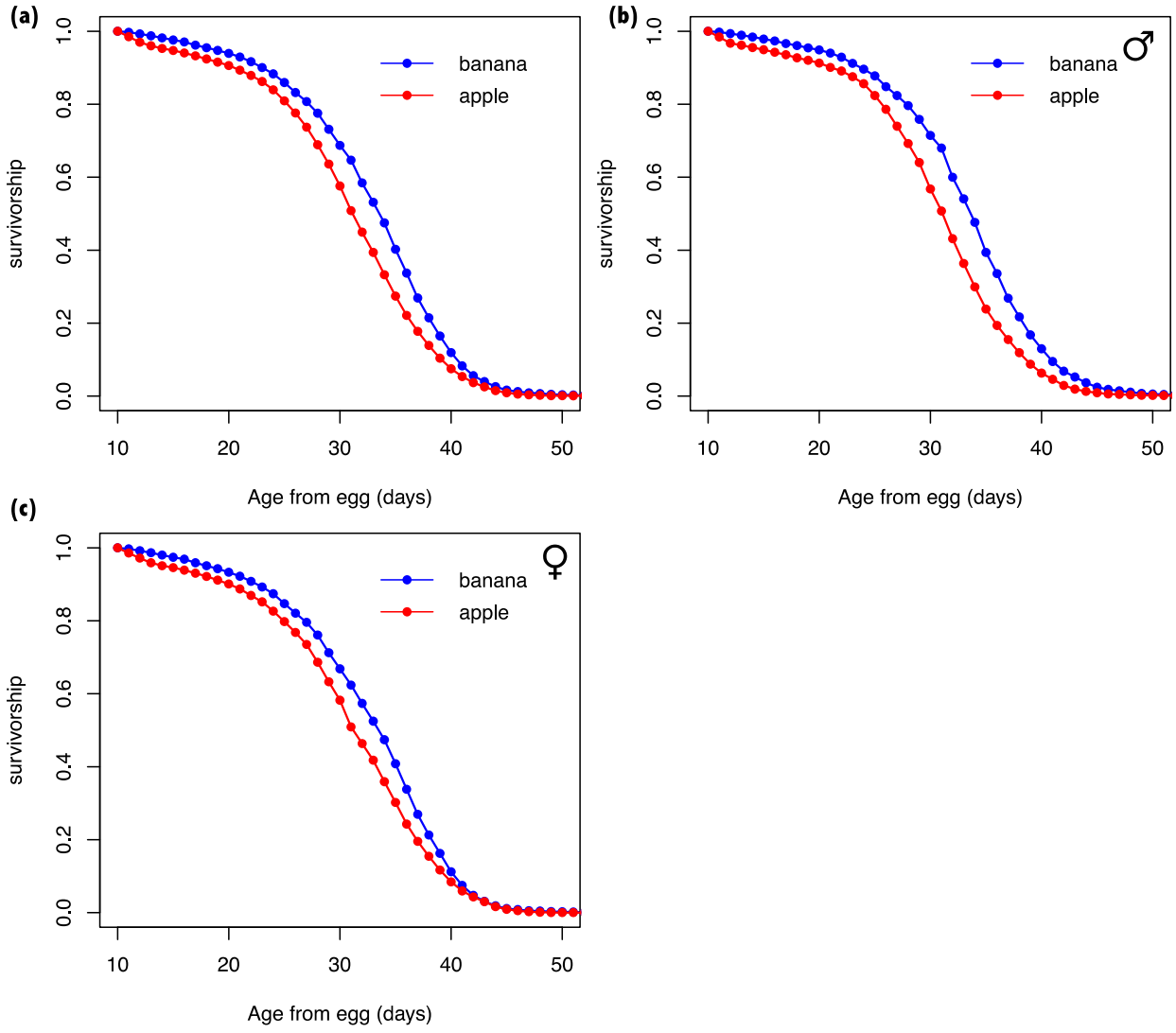


Figure S2.5 ACO survivorship for the banana and apple treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

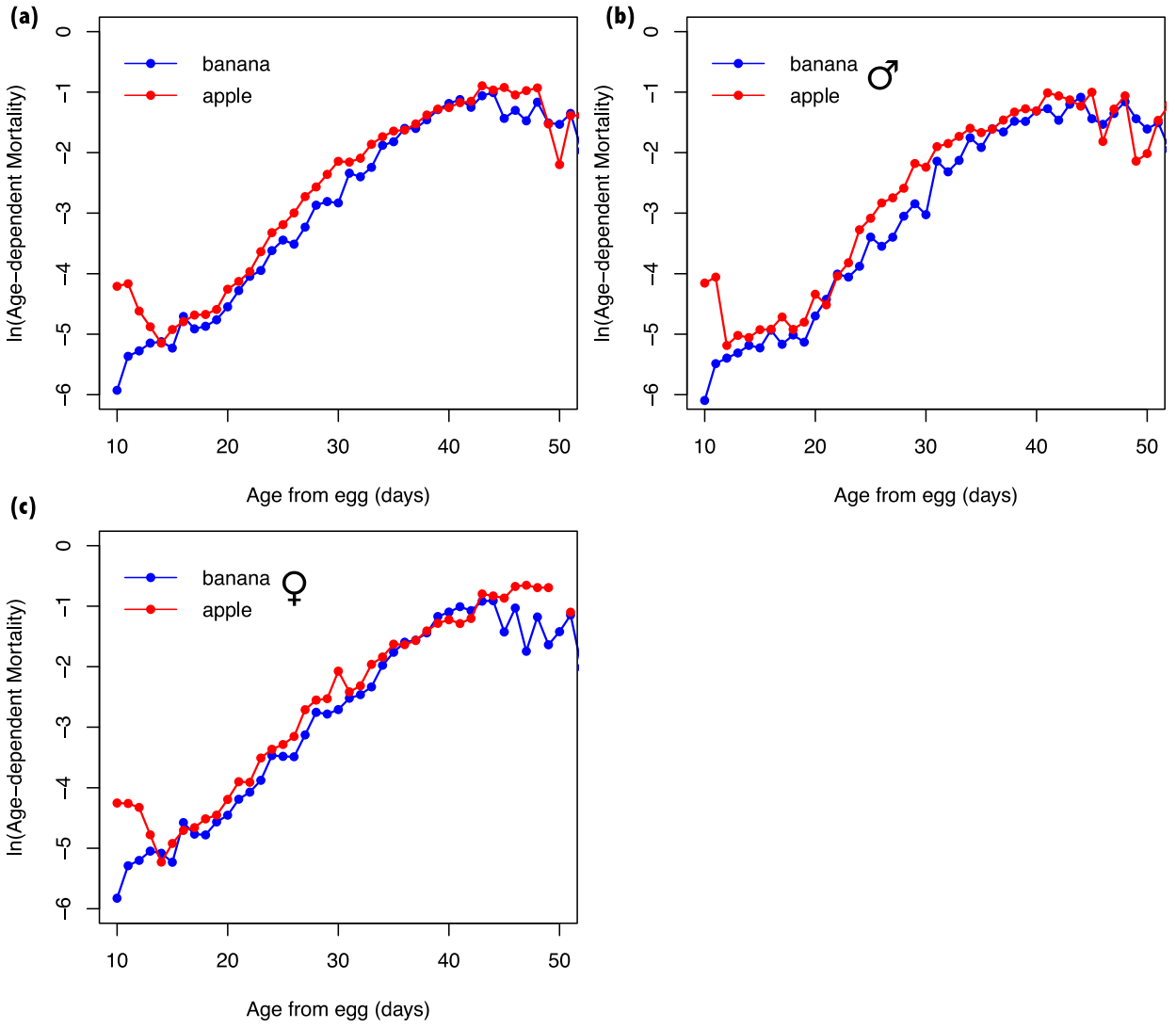


Figure S2.6 Natural log transformed age-dependent mortality for the ACO apple and banana treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

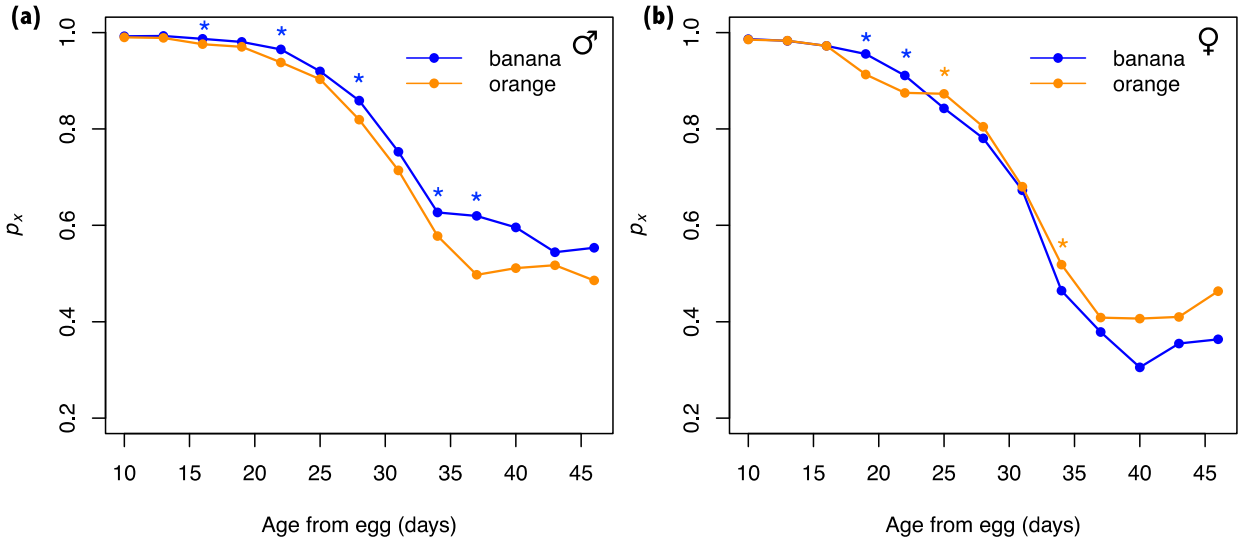


Figure S2.7 ACO conditional survival probability (p_x) over adult age from egg (x) for the banana vs. orange. (a) males and (b) females. Points represent p_x pooled across replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk signifies which treatment has higher survivorship for that interval.

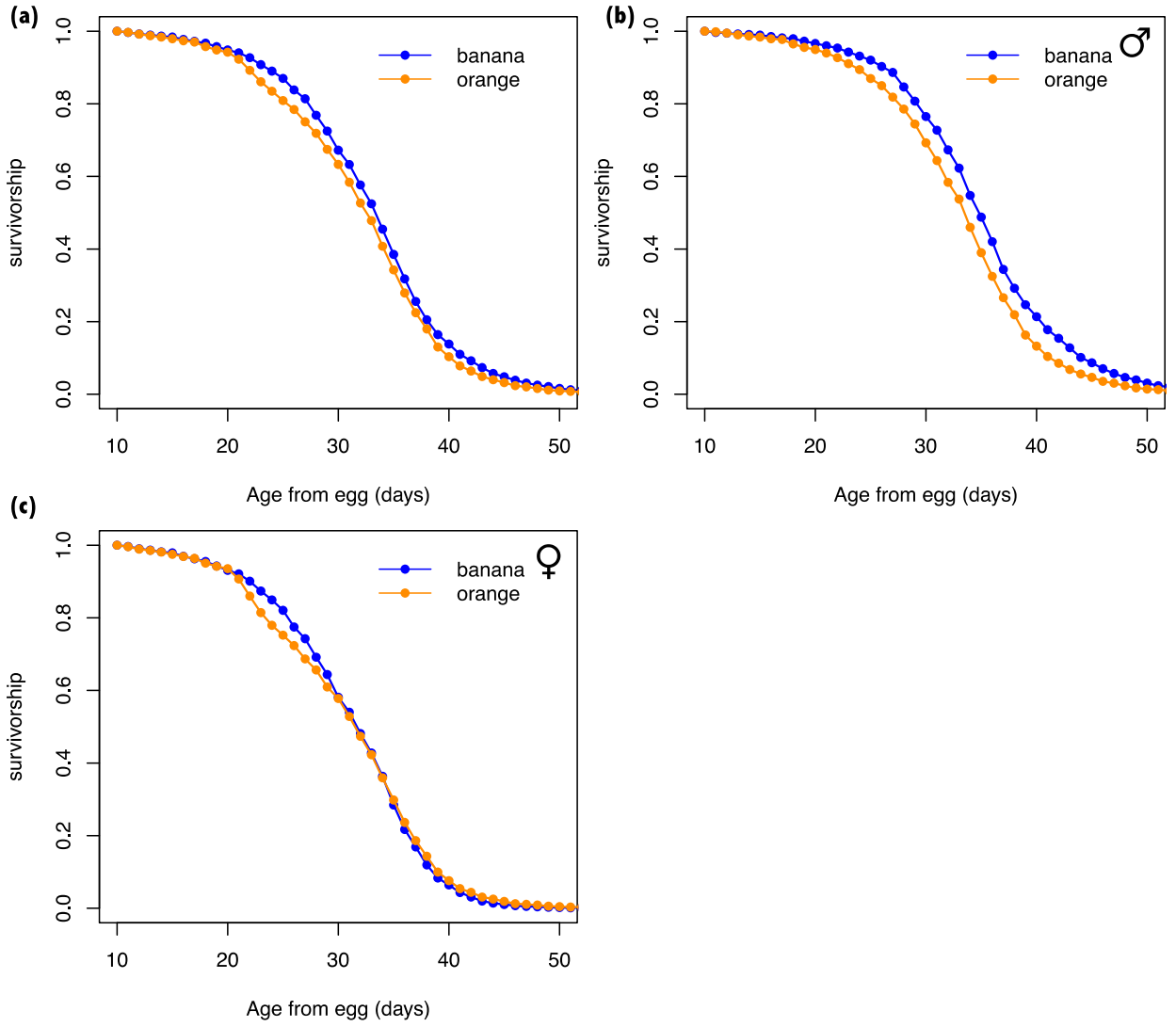


Figure S2.8 ACO survivorship for the banana and apple treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

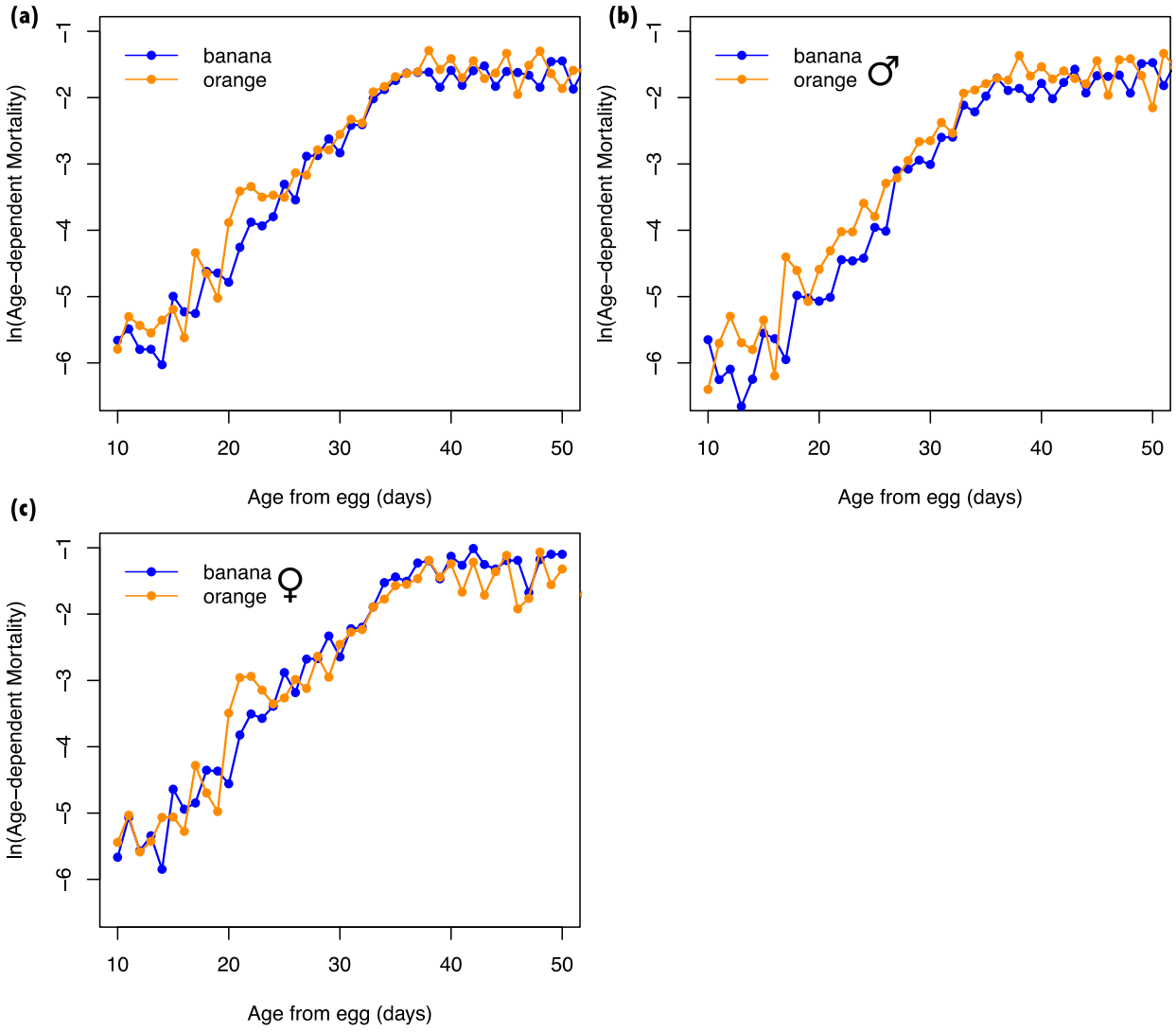


Figure S2.9 Natural log transformed age-dependent mortality for the ACO apple and banana treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

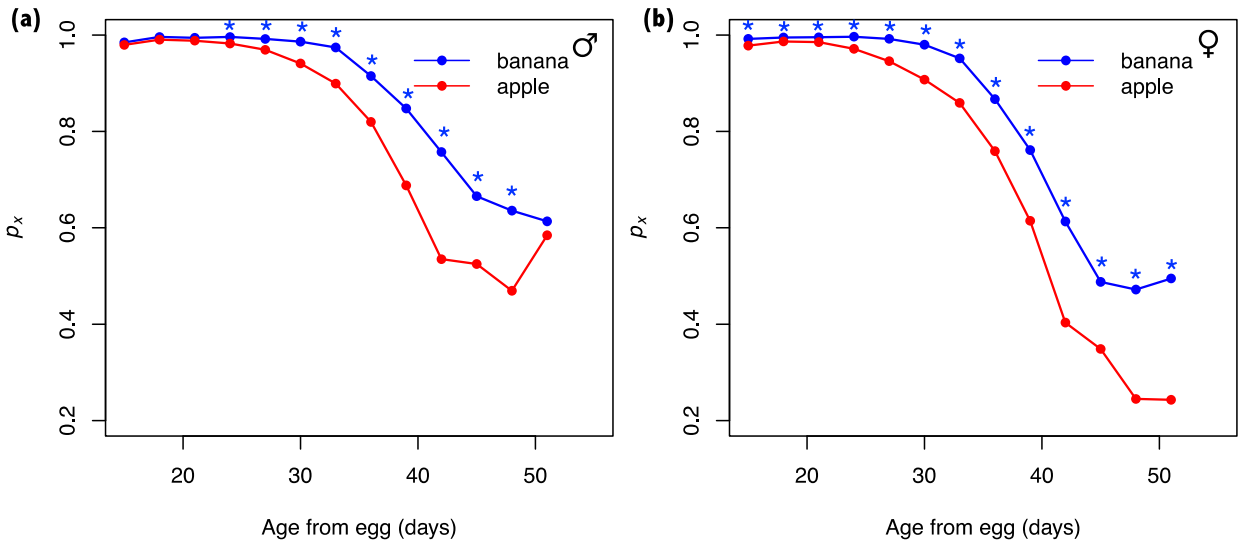


Figure S2.10 Conditional survival probability (p_x) over adult age from egg (x) for the CO banana vs. apple. (a) males and (b) females. Points represent p_x pooled across replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk signifies which treatment has higher survivorship for that interval.

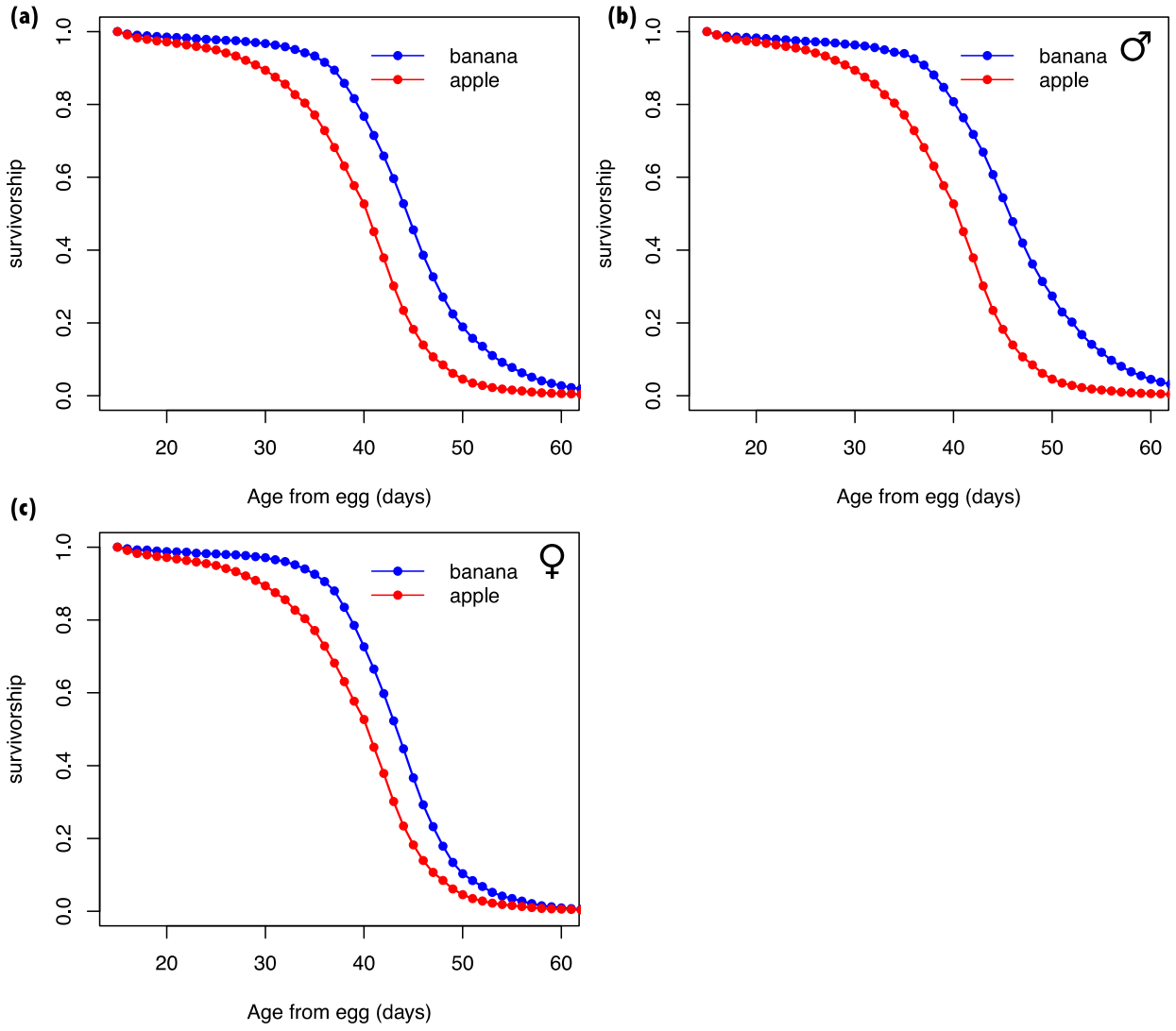


Figure S2.11 Survivorship for the CO banana and apple treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

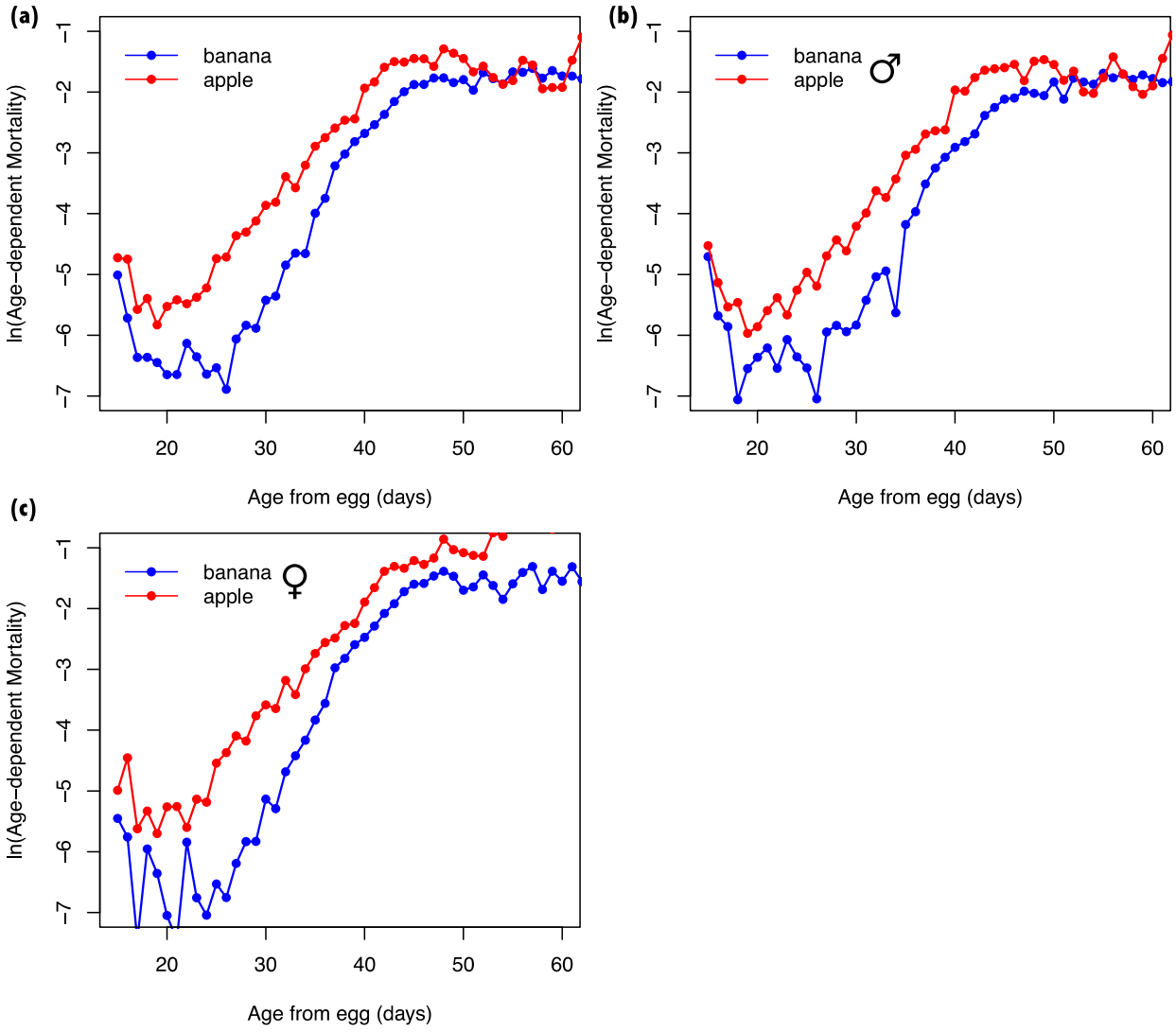


Figure S2.12 Natural log transformed age-dependent mortality for the CO banana apple treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

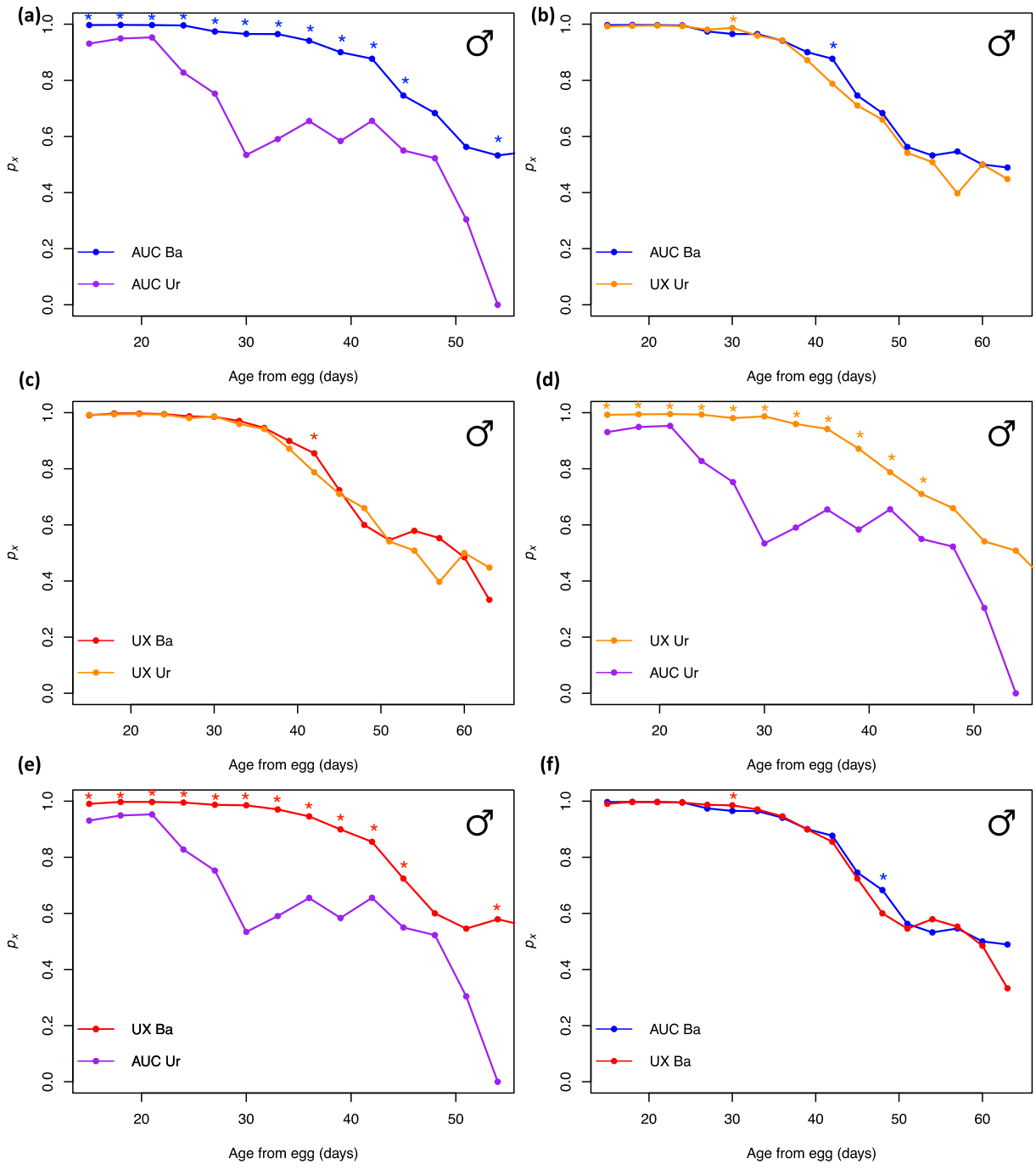


Figure S2.13 Male conditional survival probability (p_x) over adult age from egg (x) for the six comparisons in the UX/AUC experiment. (a) AUC Ba vs. AUC Ur (b) AUC Ba vs. UX Ur (c) UX Ba vs. UX Ur (d) UX Ur vs. AUC Ur (e) UX Ba vs. AUC Ur (f) AUC Ba vs. UX Ba. Points represent p_x pooled across replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk signifies which treatment has higher survivorship for that interval.

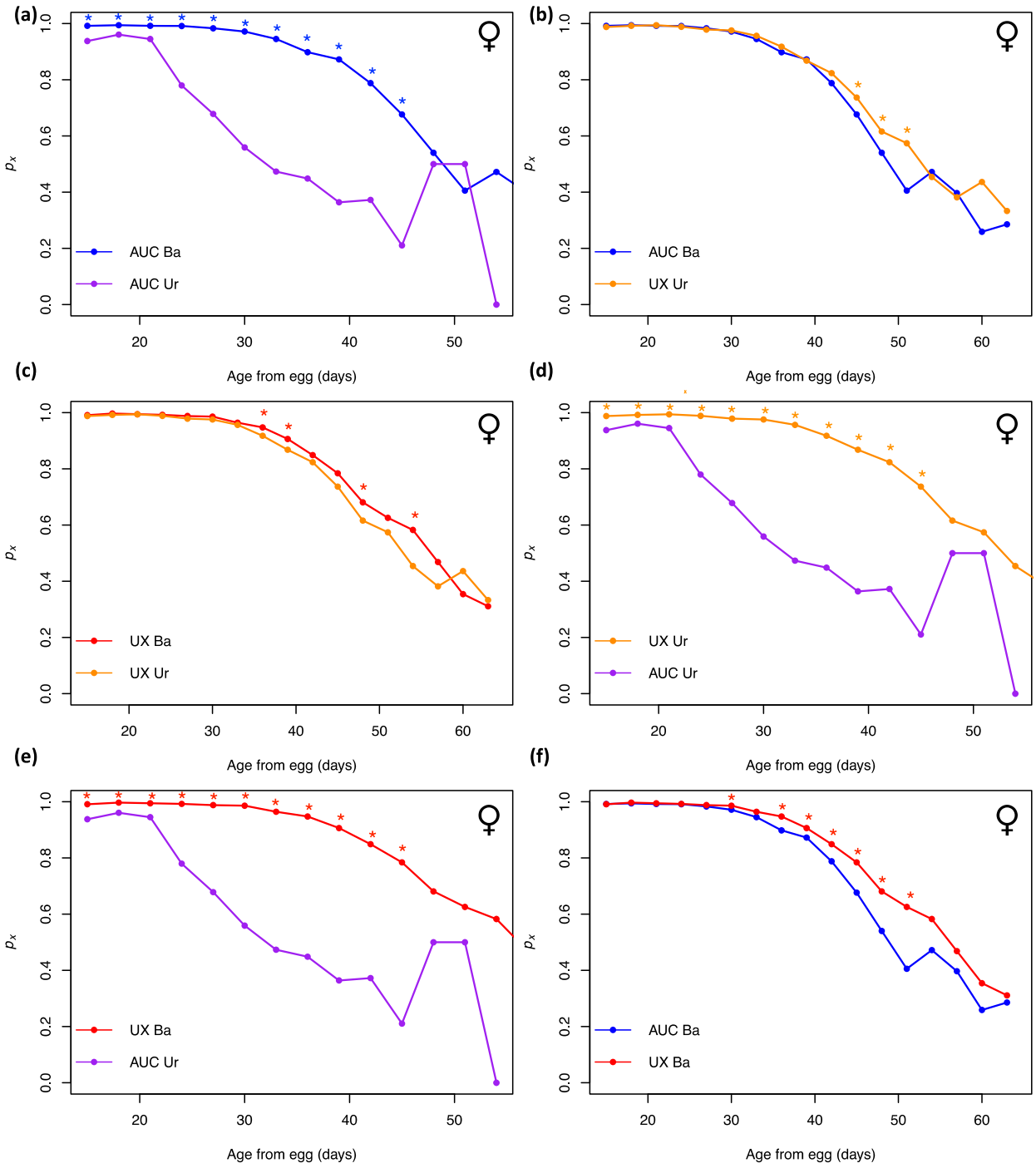


Figure S2.14 Female conditional survival probability (p_x) over adult age from egg (x) for the six comparisons in the UX/AUC experiment. (a) AUC Ba vs. AUC Ur (b) AUC Ba vs. UX Ur (c) UX Ba vs. UX Ur (d) UX Ur vs. AUC Ur (e) UX Ba vs. AUC Ur (f) AUC Ba vs. UX Ba. Points represent p_x pooled across replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk signifies which treatment has higher survivorship for that interval.

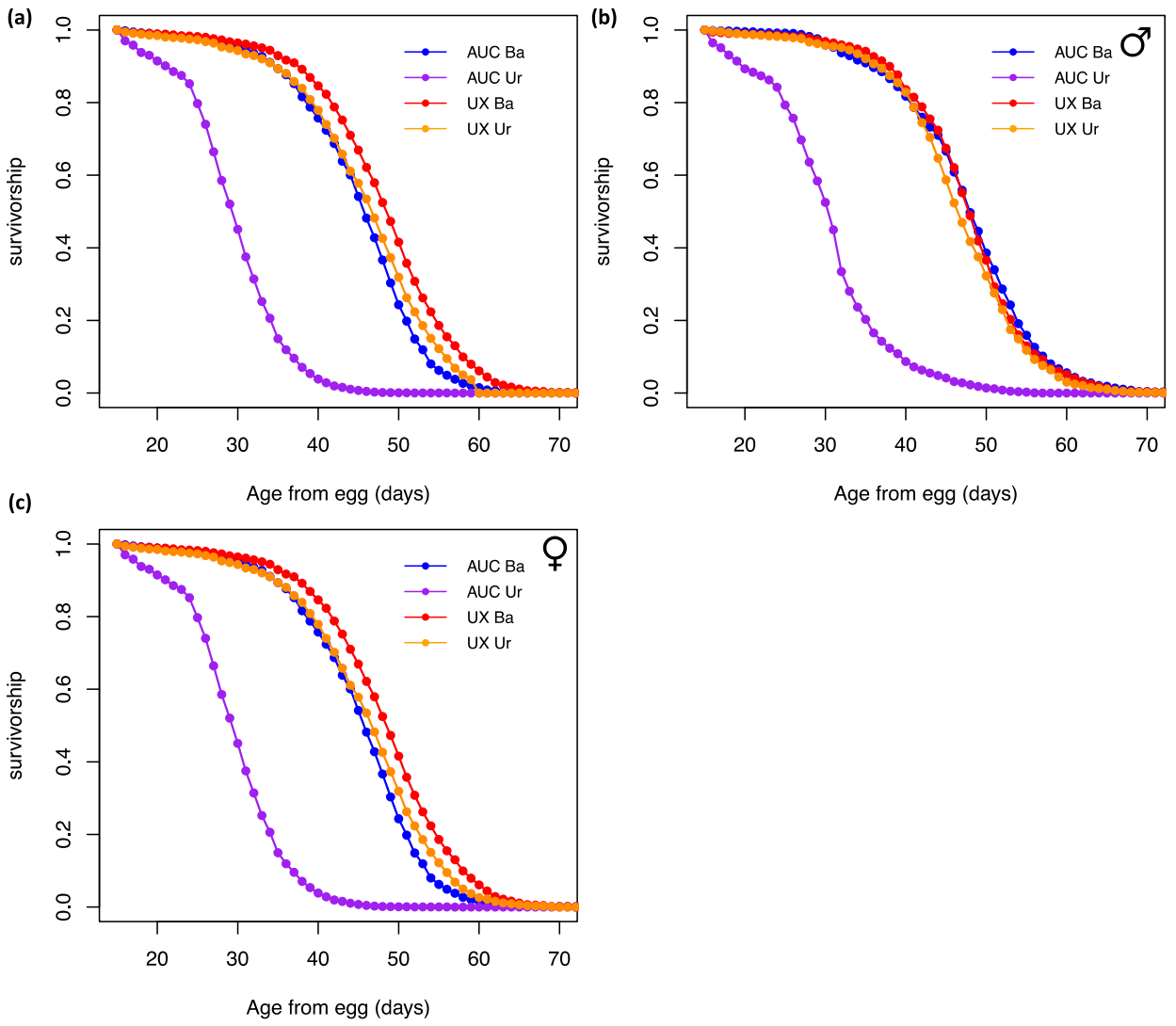


Figure S2.15 Survivorship for the AUC/UX banana and urea treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

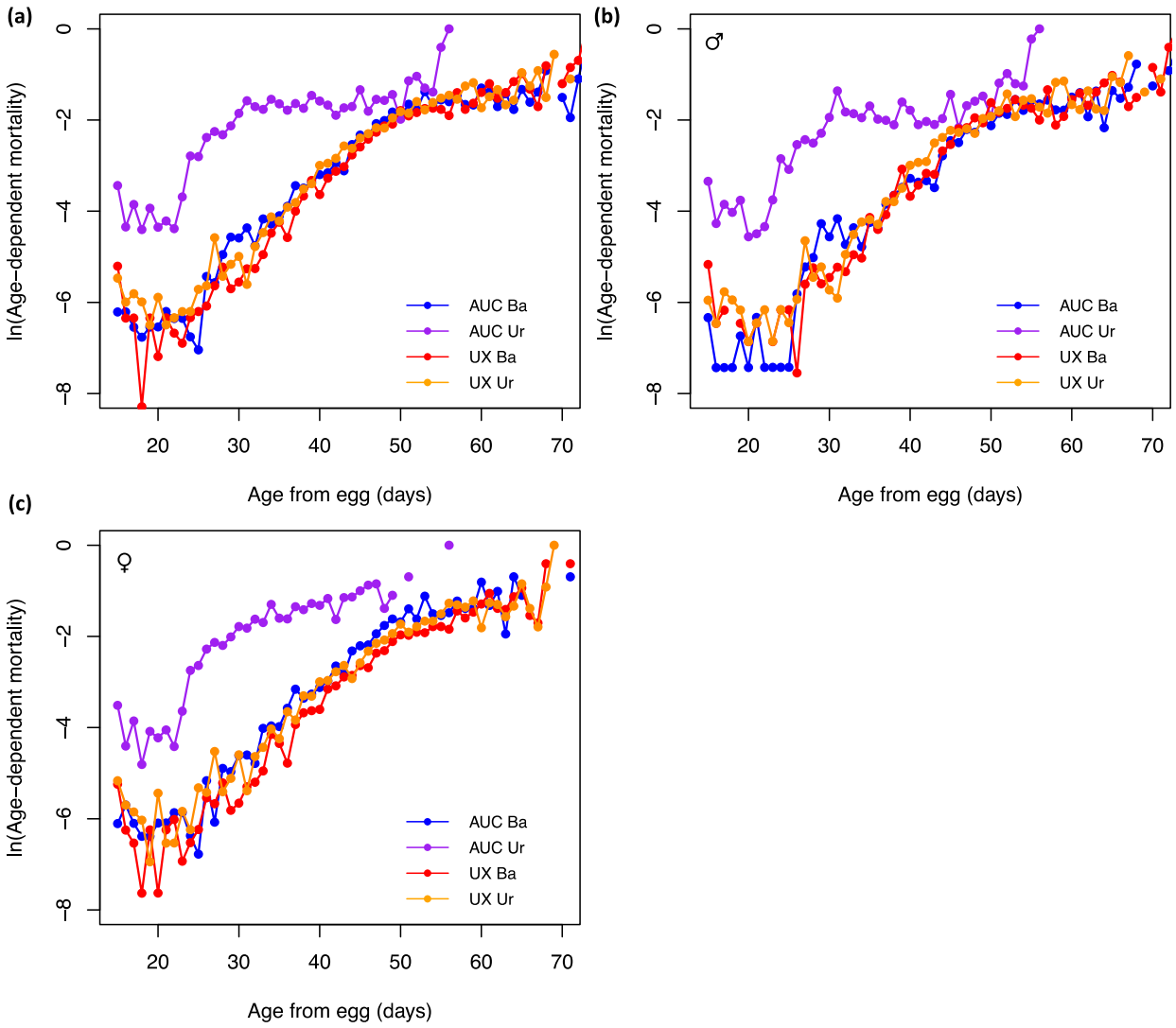


Figure S2.16 Natural log transformed age-dependent mortality for the AUC/UX banana urea treatments. (a) sexes pooled. (b) male flies. (c) female flies. Data is pooled across replicates.

CHAPTER 3

Diet and Botanical Supplementation:

Combination Therapy for Healthspan Improvement?

ABSTRACT

Healthspan science aims to add healthy, functional years to human life. Many different methods of improving healthspan have been investigated, chiefly focusing on just one aspect of an organism's health such as survivability. Studies in *Drosophila melanogaster* have demonstrated that a reversal to an ancestral diet results in improved functional health, particularly at later ages. Meanwhile, pharmaceutical studies have demonstrated that botanical extracts have potent anti-aging properties, capable of extending the mean lifespan of *D. melanogaster* by up to 25%, without a decrease in early fecundity. Here we combine these two different approaches to healthspan extension to examine if a combination of such treatments results in a synergistic or antagonistic effect on *Drosophila* healthspan. We found that one botanical extract, derived from *Rosa damascena*, decreased survivorship when combined with an ancestral diet. Another extract, derived from *Rhodiola rosea,* mimicked the later-life healthspan enhancing effects of the ancestral diet. Lastly, we see some evidence of an antagonistic effect when these botanicals are combined. These findings support the "Poisoned Chalice" hypothesis that novel combinations of supplements can elicit adverse physiological responses.

INTRODUCTION

Anti-aging studies aim to reverse or delay the effects of aging by targeting and manipulating the multiple biological pathways that cause this complex phenomenon (De Grey et al. 2006). Historically, studies of this nature have focused solely on lifespan extension, without considering the trade-offs that may affect other aspects of health (Jafari and Rose 2006). For example, though green tea supplementation has been shown in the past to increase mean lifespan of male fruit flies by 16%, recent studies reveal that it also impairs their reproductive fitness (Lopez et al. 2014). These supplements have minimal use for human application if the proposed lifespan extension results in impaired or nonfunctional health. Thus, there has been a call to shift the focus of anti-aging studies from lifespan extension to healthspan improvement (Jafari 2015). Healthspan offers a more holistic measure of an organism's health, encompassing not only lifespan but also other physiological functions that contribute to an organism's state of health. These may include, but are not limited to, reproductive fitness, locomotor activity, metabolic activity and cognitive function. By evaluating healthspan, anti-aging scientists can be sure that life extension therapies help organisms live both longer and healthier lives.

A current argument in healthspan improvement is that humans should revert to the consumption of a “Paleolithic diet” (Klonoff 2009). More specifically, it is theorized that because human ancestors consumed a hunter-gatherer diet for over 5 million years, our genome has been selected for optimal survival and reproductive fitness when consuming lean meats and natural plants, leaving modern-day humans improperly adapted to the current agricultural diet of wheat, dairy, and processed foods (Cordain et al. 2005). This diet, they suggest, has resulted in a decline in health and an increased incidence of chronic diseases such as cardiovascular disease (O’Keefe and Cordain 2004). Some investigators suggest that a reversal to the Paleolithic diet would improve

the current state of health decline by nourishing the body properly with food that the human genome had carefully adapted to through years of evolutionary selection. In fact, some findings have already proven the health benefits of reverting to this hunter-gatherer-type diet, reporting improved blood pressure, decreased LDL cholesterol and other health benefits in patients consuming the Paleolithic diet (Frassetto et al. 2009; Jönsson et al. 2009; Otten et al. 2018).

Recently, a study using *Drosophila melanogaster* populations has revealed that a reversal to an ancestral diet results in healthspan improvement, but in an age-specific manner (Rutledge et al. in prep). In this study, laboratory fly populations were fed two different diets: banana food and apple food. These laboratory flies had lived on banana molasses food for over 30 years since they were collected from the wild (~1000 generations). Prior to laboratory domestication, these flies consumed rotting apples in an apple orchard in the northeastern United States for centuries (Ives 1970). Thus, banana food represents their evolutionarily recent diet, and apple food represents their ancestral long-standing diet. Healthspan was measured using age-specific assays of female survivorship probability (p_x) and fecundity (m_x), which when multiplied together ($p_x m_x$) provides a healthspan curve over all adult ages (See Materials and Methods) (Rutledge and Rose 2015). The results showed that $p_x m_x$ remained relatively equal in the banana and apple fed flies at early ages with some evidence of a decrease in age-specific survivorship in early ages of adult life among apple fed flies. At later ages, the apple group began to show superior $p_x m_x$. These data indicate that the benefits of reverting to a long-abandoned ancestral diet may manifest only at later ages.

This finding was not unexpected. Our intuitive understanding of adaptation by natural selection is dominated by the power of selection at early ages in large outbred populations (Hamilton 1966). But, as the forces of natural selection fall with adult age, we expect adaptation to decline with age. This suggests that populations should adapt to a novel environment quickly at

early ages, but slowly and incompletely at later ages (Rose et al. 2014). This attenuation in the forces of natural selection should result in populations being better adapted to an abandoned ancestral diet particularly at later ages. However, populations should be able to achieve reasonable health on a more recently imposed, though sufficiently longstanding, diet at younger ages. Whether populations are better off consuming a recently imposed diet, or a long-abandoned ancestral diet at early ages remains unclear.

Meanwhile, Jafari and colleagues have been researching anti-aging botanical extracts known to increase lifespan without adversely affecting other healthspan measurements. Two botanical extracts derived from *Rhodiola rosea* and *Rosa damascena*, have been shown to extend mean lifespan by 25% and decrease mortality by 22%, respectively (Jafari et al. 2007; Jafari et al. 2008). Both botanical extracts extended life without causing significant impairment to physiological functions, including fecundity. Though fecundity was not measured throughout the entire fly lifespan, 10-day dose-response assays were conducted for both botanicals to investigate any impairment of reproduction. Over a course of 10 days, various doses of *Rhodiola rosea* and *Rosa damascena* did not significantly affect fecundity in female flies (Jafari et al. 2007; Jafari et al. 2008). However, these data did not demonstrate whether later-age fecundity in *Drosophila* is affected by the botanicals.

The studies performed in both labs yielded complementary results; Rutledge et al. (in prep.) demonstrated healthspan improvement via increased later-age fecundity with an ancestral diet, while Jafari and colleagues demonstrated healthspan improvement via increased lifespan with botanicals. This study will evaluate the effects of combining both these anti-aging techniques. In addition, we will study the effects of combining multiple botanicals on healthspan. This second idea was inspired by a recent review on multi supplementation (Rutledge and Rose 2015).

Numerous studies using humans and mice have not found combined supplementation to be useful for preventing diseases and decreasing mortality (Macpherson et al. 2013; Watkins et al. 2000; Park et al. 2011; Spindler et al. 2014). In fact, Spindler et al. (2014) found that some complex nutraceuticals decreased lifespan in mice. More recently, Jenkins et al. (2018) performed a meta-analysis in humans and found that antioxidant mixtures and niacin resulted in an increase in all cause-mortality. Rutledge and Rose (2015) coined the "Poisoned Chalice" effect as the reason for the lack of success in these studies, suggesting that excessive multi supplementation could interfere with an organism's complex network of biochemical reactions built through evolutionary refinement of positive and negative feedback controls. Thus, individual substances that alone improve healthspan may have a negative impact on healthspan when combined.

In this study we will investigate in *Drosophila melanogaster* the effectiveness of *Rosa damascena* in two diet backgrounds researched extensively by Rutledge and colleagues in a large-scale, well-replicated, full lifespan study. If a long-abandoned diet improves later-age fecundity, and if the botanical *Rosa damascena* increases lifespan without negatively impacting healthspan, then a combination of these two treatments should result in improved healthspan at a magnitude larger than observed from either treatment individually. Otherwise, there is evidence for a Poisoned Chalice effect. Additionally, if *Rosa damascena* and *Rhodiola rosea* both increase lifespan separately, then combining these botanicals could result in improved lifespan at a larger magnitude than observed from either treatment alone, or again we would have evidence for a Poisoned Chalice effect.

MATERIALS AND METHODS

Study system: We used large, outbred populations of *Drosophila melanogaster* selected for accelerated development (Chippindale et al., 1997). Five "ACO" replicates (ACO_{1-5}) have been reared on banana-molasses food for ~1000 generations and have a 10-day life cycle. The wild *Drosophila* population from which these laboratory populations were derived is that of Northeastern United States; the local agricultural setting is one that has featured apples as the chief cultivated fruit for centuries (Ives, 1970). A more detailed and up-to-date description of the history and culture methods for these lines can be found in Burke et al. (2016).

Overall experimental design: Two experiments were performed that monitored time-to-death (mortality) and 24-hour fecundity. In one experiment, ACO_{1-5} were exposed to four treatments: banana food with *Rosa damascena* supplementation, banana food without *Rosa damascena* supplementation, apple food with *Rosa damascena* supplementation, and apple food without *R. damascena* supplementation. In another experiment, ACO_{1-5} were exposed to four different treatments: banana food (control), banana food with *Rosa damascena* supplementation, banana food with *Rhodiola rosea* supplementation, and banana food with the combination of both *Rosa damascena* and *Rhodiola rosea*. This second experiment was performed with the banana-molasses diet only and will be referred to as the "combination experiment".

Food preparation: ACO populations were reared on a banana-molasses diet for stock maintenance and for select experimental assays. The banana-molasses media is composed of the following ingredients per 1L distilled H_20: 13.5g Apex® Drosophila agar type II, 121g peeled, ripe banana, 10.8mL light Karo® corn syrup, 10.8mL dark Karo® corn syrup, 16.1mL Eden®organic barley malt syrup, 32.3g Red Star® active dry yeast, 2.1g Sigma-Aldrich® Methyl 4-hydroxybenzoate (anti-fungal), and 42.5 mL EtOH. The apple media is prepared in the same

manner as the banana media, except the diet lacks the barley malt, corn syrups, and we substitute 1 to 1 Trader Joes® organic unsweetened apple sauce for the peeled banana.

Supplement administration: Populations were administered extracts as described by Jafari et al. (Jafari et al., 2007; Jafari et al., 2008). The *Rhodiola rosea* (SHR-5) root extract of 1.42% salidroside and 3% rosavins, as characterized by HPLC, was administered in 3% yeast solution at a 25 mg/mL dosage. The *Rosa damascena* petal extract, prepared and obtained from Dr. Asghar Zarban, was administered in 3% yeast solution at a 2 mg/mL dosage. Both concentrations have been experimentally reported by Jafari et al. as the ideal biological dosage. The combination treatment of *Rhodiola rosea* and *Rosa damascena* was a combined dose of 25 mg/mL and 2mg/mL respectively. Each food plate received 1 mL of yeast solution, and each cage received fresh food and supplement every 24 hours. Supplement was not administered during the developmental stage (day 1-9 from egg).

Mortality and fecundity assays: Populations were initially reared in 8-dram polystyrene vials with ~6mL of either banana food or apple food at ~70 eggs/vial and given 9 days to develop. Flies were then transferred into acrylic cages using light carbon dioxide anesthetic and given fresh food with the respective treatment. Individual mortality was assessed every 24 hours, the flies were sexed at death, and the observed cohort size was calculated from the complete recorded deaths. During the assay, flies were transferred to clean cages once a week using light CO_2 anesthesia. Cohorts were assayed in 6L cages at ~1000 flies per cage. Flies were transferred to 3L cages at 50% starting cohort size to control for density effects. Age-specific fecundity was also assessed every 24-hours. This parameter was estimated from the number of eggs laid by females on the culture medium plates placed in each mortality assay cage, divided by the number of females still alive. Media plates were washed on filter paper with the lab's fecundity funnel system and then

scanned for counting at a later time (Burke et al., 2016). Egg counting was performed using ImageJ (https://imagej.nih.gov/ij/), a National Institute of Health validated image-processing program.

p_xm_x statistical analysis: The age-specific survival probability *(p_x)* is the probability of a female surviving to age *x,* given that she survived to the start of the age-interval. It is calculated using the following equation:

$$p_x = 1 - \left(\frac{d_x}{n_x}\right)$$

where d_x is the number of females that die at age *x,* and n_x is the number of females that were alive at the start of age *x*. Age-specific fecundity (m_x) is the average number of eggs laid per surviving female at age *x*. The product of these two variables gives an estimate of how cohorts are functioning at each age. In our experiments, the unit interval for *x* is a single day. We will refer to this parameter as age-dependent fitness or health.

We tested for differences in p_xm_x in 13, three-day age-classes (day from egg 10-12, 13-15…). The observations consisted of p_xm_x at an age (x) within an age interval (k = 1, 2,…,13). Within each interval, p_xm_x was modeled by a straight line allowing diet (j=1 for banana, j=2 for apple, *j=3* for apple *Rosa damascena* or *j= 4* banana *Rosa damascena*) to affect the intercept, but not the slope of the line. Slope could vary between intervals. Populations (i = 1, 2…,20) contributed random variation to these measures. With the notation above, the p_xm_x at age (x), interval (k), diet (j), and population (i) is y_{ijkx} and can be described by,

$$y_{ijkx} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)x + \delta_k\delta_j\mu_{jk} + c_i + \mathcal{E}_{ijkx},$$

where $\delta_s = 0$ if s = 1 and 1 otherwise, and c_i and $\mathcal{E}_{ijkx}$ are independent standard normal random variables with variance σ_c^2 and $\sigma_\mathcal{E}^2$, respectively. The effects of diet on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}. Statistical computing was completed in R (https://www.r-project.org/) using the Linear and Nonlinear Mixed Effects (nlme)

package. The Least-Squares means (lsmeans) package in R was used to calculate p-values from the multiple comparisons. A Tukey's range test was used to correct for multiple comparisons. A *p*-value less than 0.05 was considered statistically significant. The data from the combination experiment was analyzed using the same method.

p_x age-specific survivorship analysis: For each combination of *treatment*sex* three-day survivorship intervals were computed. For each interval a new categorical variable was then created, defining the status of each one of the flies (0 = dead or 1 = alive). The counts of each interval were used in a chi-squared test to compare all treatment combinations in both the *Rosa damascena* experiment and the combination experiment. A Bonferroni correction was applied to correct for the multiple age-classes per comparison. Analysis was completed with the "survival" package in R (https://www.r-project.org/). A *p*-value of less than 0.05 was considered statistically significant.

Fecundity statistical analysis: Average eggs per surviving female (m_x) was analyzed using a paired t test with replicates 1-5 treated as pairs across treatments analyzed. Early fecundity comprised the average of the first half of the assay (~20 days) and later fecundity comprised of the average of the second half of the assay (~20 days). Average fecundity comprised of the average of the entire assay (~40 days). A *p*-value less than 0.05 was considered statistically significant.

Mean Longevity analysis: Mean longevity was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org/). The model used for the data is described as follows: Let y_{ijkm} be the longevity for diet – i (i =1 (banana), 2 (apple), 3 (banana *Rosa damascena*), 4 (apple *Rosa damascena*)), sex-j (j=1 (female), 2(male)), cage – k (k=1,.., 40) and individual – m (m=1,.., n_{jk}). A LME model for longevity is,

$$z_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where $\delta_s = 0$, if s = 1, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively. The combination experiment used the same model as above but with different diets-i.

RESULTS

Rosa damascena Supplementation Experiment

Age dependent fitness (p_xm_x) analysis

When observing p_xm_x for the apple vs. apple *Rosa damascena* treatments, we see no differences at any of the 13 intervals (Fig. 1b, Table S2). This is also generally the case for the banana vs. banana *Rosa damascena* treatments, except banana is statistically higher than banana *Rosa damascena* for one interval early in the assay (Fig. 1c, Table S3). Flies exposed to either the apple treatment or the apple *Rosa damascena* treatment generally show lower p_xm_x at early ages and higher p_xm_x at later ages when compared to the banana or the banana *Rosa damascena* treatment. (Figure. 3.1a, 3.1d-f; Table S3.1, S3.4-S3.6). We see the greatest late-life p_xm_x enhancement in the apple *Rosa damascena* treatment when compared to the banana or banana *R. damascena* treatment (Figure 3.1d-e).

Age-specific survival (p_x) and mean longevity analysis

When analyzing conditional survival probability (p_x), we see the strongest effects in female flies (Figure 3.2). Reponses in males are generally much weaker or non-existent (Figure S3.1). Female survival is significantly higher on the apple diet compared to the banana diet for most intervals (Figure 3.2a). However, notably the first interval is consistently higher in the treatments with the banana background compared to the apple background (Figure 3.2). Female mean longevity is significantly longer on the apple diet compared to the banana diet (p<0.001; Table 3.1). In the banana fed flies, female survival is significantly higher with *Rosa damascena* supplementation for several intervals (Figure 3.2c). Female mean longevity increase is also weakly significant in the banana *Rosa damascena* (p=0.0469; Table 3.1). Interestingly, this trend is opposite with apple fed flies. In the apple-fed flies, female survival is significantly higher with the apple compared to the apple *Rosa damascena* for several intervals (Figure 3.2b). However, mean

longevity did not differ significantly (Table 3.1). This contrast is clear when observing the survivorship curves and to a lesser extent the mortality curves for these treatments (Figure S3.2-S3.3).

Fecundity (m_x) analysis

Rosa damascena supplementation does not significantly affect average fecundity in both the banana and apple diet backgrounds (Figure 3.3b-c). For banana, later fecundity is significantly reduced (p=0.035), however early fecundity is not significantly affected (p>0.05). Generally, the apple diet with or without *Rosa damascena* supplementation enhances later life fecundity and reduces early life fecundity compared to the banana diet with or without *Rosa damascena*.

Rhodiola rosea and *Rosa damascena* Combination Experiment

Age-dependent fitness (p_xm_x) analysis

When *Rhodiola rosea* is supplemented, we see a general trend of lower p_xm_x at early ages and higher p_xm_x at later ages when compared to the control, or *Rosa damascena* treatments (Figure 3.4a, d-f; Table S3.7, S3.10-3.12). This is like the trend we see in the apple versus banana treatments, but p_xm_x is more reduced at earlier ages in the *Rhodiola rosea* treatments (Figure 3.1; Figure 3.4). When observing p_xm_x for the control compared to the *Rhodiola rosea* treatment, we see significantly higher p_xm_x at early ages in the control and significantly lower p_xm_x at later ages (Figure 3.4a; Table S3.7). When comparing the control to the *Rosa damascena* treatment, we see no difference in p_xm_x at any of the intervals (Figure 3.4b; Table S3.8). In addition, we see no difference in *Rhodiola rosea* supplementation vs. the combination treatment (Figure 3.4c; Table S3.9).

Age-specific survival (p_x) & mean longevity analysis

When analyzing age-specific survival probability (p_x), we see the greatest difference in survival in males and females from the *Rhodiola rosea* and combination treatments compared to the control and *Rosa damascena* treatments (Figure 3.5, S3.4). This contrast is clear when observing the survivorship curves and mortality curves for these treatments (Figure S3.5-S3.6). *Rosa damascena* does increase survival significantly in some intervals in the males (Figure S3.4b) and one interval in the females (Figure 3.5b), however its effectiveness in increasing survival is not as strong as the *Rhodiola rosea* (Figure 3.5e, S3.4e). Mean longevity is significantly increased in males and females of the *Rhodiola rosea* treatment compared to the control and *Rosa damascena* treatments (Table 3.2). Mean longevity is not significantly increased in the *Rosa damascena* treatment compared to the control (Table 3.2). Interestingly, we see survival is significantly decreased in a few of the later intervals of the combination treatment compared to the *Rhodiola rosea* (Figure 3.4c, S3.4c), however this difference is slight. Mean longevity for this comparison is reduced in the combination treatment, albeit it is not significant (Table 3.2).

Fecundity (m_x) analysis

When analyzing fecundity, the treatments with *Rhodiola rosea* supplementation have significantly lower early m_x compared to the control and *Rosa* damascena treatments (Figure 3.6). This dramatic decrease in earlier fecundity is to blame for the significant decrease in average m_x for these treatments. However, later fecundity is not significantly different. Fecundity for the last 10 days of the fecundity assay is likely to be enhanced in the *Rhodiola rosea* treatments compared to the control and *Rosa damascena.* This is evident when observing the p_xm_x graphs for these comparisons (Figure 3.4). There does not appear to be a decrease in fecundity in the *Rosa*

damascena treatment compared to control (Figure 3.6b). Lastly, no fecundity difference exists between the *Rhodiola rosea* and combination treatments (Figure 3.6c).

DISCUSSION

In this study we were able to reproduce the results of Rutledge et al. (in prep.). Flies on the banana diet had a significantly higher $p_x m_x$ at early ages and significantly lower $p_x m_x$ at later ages, compared to flies on the apple diet. The effect of the apple diet at later ages was observed in that study, but the clear decrease in $p_x m_x$ at early ages on the apple diet found here was not shown. These results suggest that the ideal diet for younger individuals may not be the same as the ideal diet for older individuals. Humans with agricultural ancestry may achieve better health while consuming an organic agricultural diet at earlier ages, and a more paleolithic diet at later ages, compared to individuals who consume a strictly paleo diet throughout life. In addition, the apple-fed female flies lived ~14% longer than the banana fed flies.

We were also able to partially reproduce the *Rosa damascena* results from Jafari et al. (2008) in a large cohort study with ~13,000 flies per treatment. Using the same background diet as was used in that study (banana-molasses medium), we found that females had significantly higher survivorship for days 22-40 from egg when given *Rosa damascena*. In addition, female mean longevity increased significantly (~5%). Notably, we see an increase in longevity and survivorship without a significant decrease in average female fecundity. However, $p_x m_x$ was not significantly different between the banana control flies and the *Rosa damascena* supplemented flies for any of the age-classes. Interestingly, female survivorship in the apple fed flies was significantly lower for five age-classes (15 days) when the apple food is supplemented with *Rosa damascena*. This finding suggests that the lifespan extension with *Rosa damascena* not only depends on diet but combining substances with survivorship enhancing properties does not necessarily produce an additive effect. In fact, the interaction may be detrimental to the organism, as is observed with our study for survivorship.

On the other hand, when the apple food is supplemented with *Rosa damascena* we see an increase in $p_x m_x$ at later ages when compared to the banana food. This late-life increase in $p_x m_x$ is stronger when *Rosa damascena* is present in the apple food than when it is not. However, no difference is seen when we compare apple to apple *Rosa damascena*.

Supplementation with *Rhodiola rosea* produced a similar life-extending effect as was found in Jafari et al. (2007). *Rhodiola rosea* significantly extended lifespan by 16% in males and 18% in females. Survivorship was higher for almost every age-class in both males and females. However, contrary to the early fecundity result that was found in Jafari et al. (2007), early fecundity is dramatically reduced ($p<0.001$). That study did find reductions in fecundity, but only at doses four times higher than the dose used in our study (Jafari et al. 2007). Average fecundity was also significantly lower in the *Rhodiola rosea* treated flies, but later fecundity was not affected. One possibility is that this significant early fecundity effect arose with this particular "batch" of *Rhodiola rosea.* Another confounding factor is that the assay of Jafari et al. (2007) was conducted in vials, while the present study's fecundity assay was performed in population cages. Despite this significant decrease in female fecundity, the trend in $p_x m_x$ is quite similar to what is observed in our apple control vs. banana control. $p_x m_x$ is significantly higher in the control at early ages and lower at later ages, compared to *Rhodiola rosea*. This switch to better performance in the *Rhodiola rosea* occurs around 38 days from egg, as is observed in the apple diet. In addition, we found significantly higher survivorship and longer mean longevity in the apple diet and *Rhodiola rosea* treatment compared to the banana.

Lastly, we observed some evidence of a Poisoned Chalice effect occurring in the combination of *Rhodiola rosea* and *Rosa damascena*. This occurs particularly in survivorship with

female flies, with *Rhodiola rosea* supplementation outperforming flies on the combination for three later age intervals (Figure 5c). This effect was not observed in $p_x m_x$ or fecundity. This will remain an active area of research in our lab, with more supplement combinations to be studied.

CONLCUSION

In our study, we were able to successfully reproduce the lifespan extending effects of *Rhodiola rosea* and *Rosa damascena* on a large scale with ~13,000 outbred flies used per treatment rather than hundreds. Our results indicate that supplementing a healthspan-extending diet (apple food) with a healthspan extending botanical supplement (*Rosa damascena*) does not result in an additive beneficial effect. In fact, a negative interaction may occur, resulting in a decrease in survivorship. The effect of *Rhodiola rosea* on healthspan mimics the effect of the ancestral, apple, fly diet with $p_x m_x$ significantly lower at early ages and higher at later ages. Lastly, combining botanical supplements may result in negative effects on healthspan. More experiments on the effects of combining promising healthspan-extending substances with other promising substances in various diet backgrounds is an obvious next step for this line of work.

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FIGURES

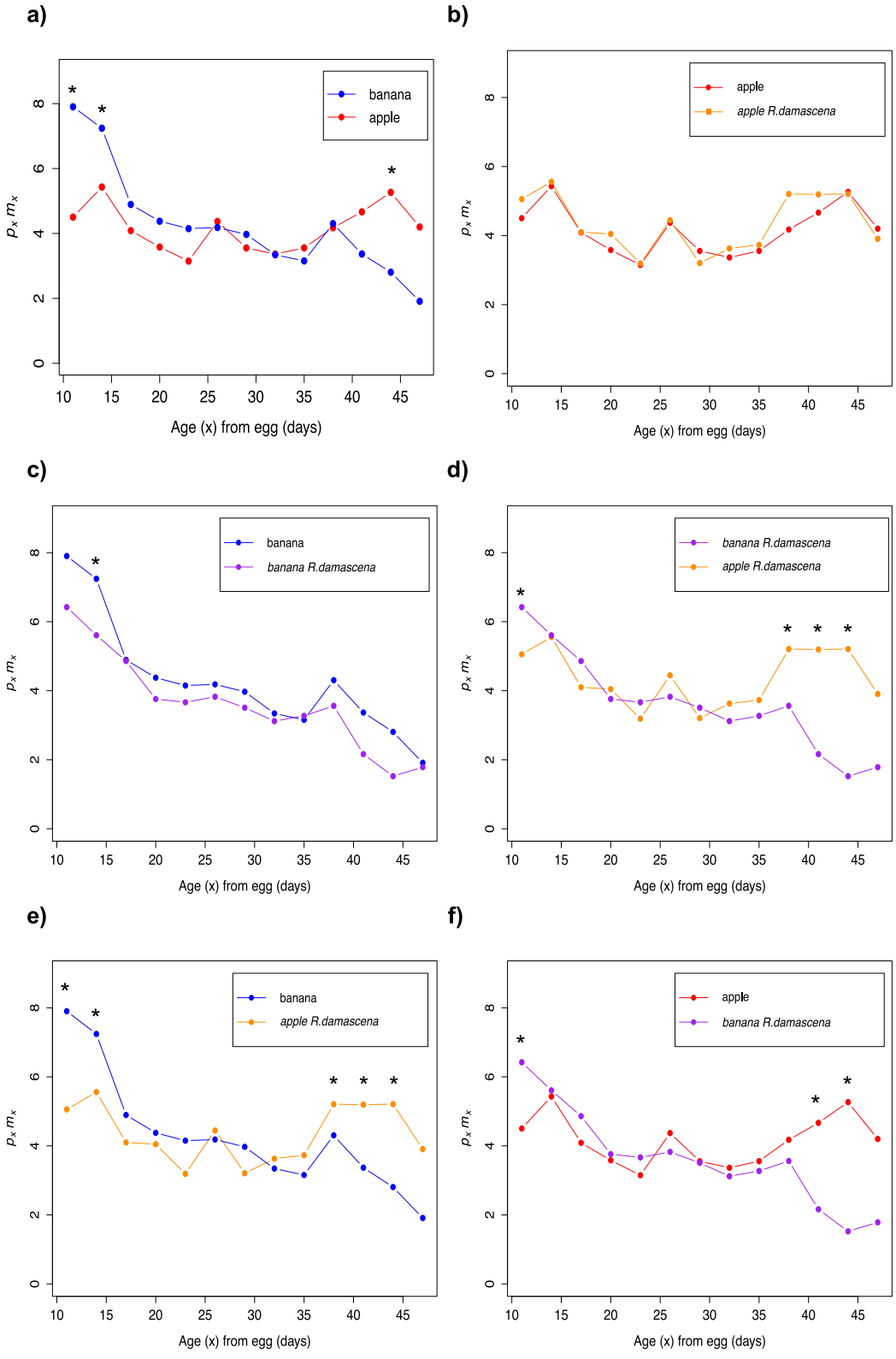

a)
b)
c)
d)
e)
f)
banana
apple
apple R.damascena
banana R.damascena
$p_x m_x$
Age (x) from egg (days)

Figure 3.1 Age-dependent fitness ($p_x m_x$) over adult age (x) for the *Rosa damascena* (*R. damascena*) supplementation experiment. (a) The banana treatment is generally higher at early ages and lower at later ages compared to the apple treatment. **(b)** No difference is observed between apple and apple *Rosa damascena* at any age. **(c)** Banana *Rosa damascena* is significantly lower for only one interval early in adulthood compared to the banana. **(d)** The banana *Rosa damascena* treatment is generally higher at early ages and lower at later ages compared to the apple *Rosa damascena.* **(e)** The banana treatment is generally higher at early ages and lower at later ages compared to the apple *Rosa damascena* treatment. **(f)** The banana *Rosa damascena* treatment is generally higher at early ages and lower at later ages compared to the apple treatment. Points represent the pooled data across the five replicates averaged across three days. *denotes significance for that interval between shown diets (p<0.05)

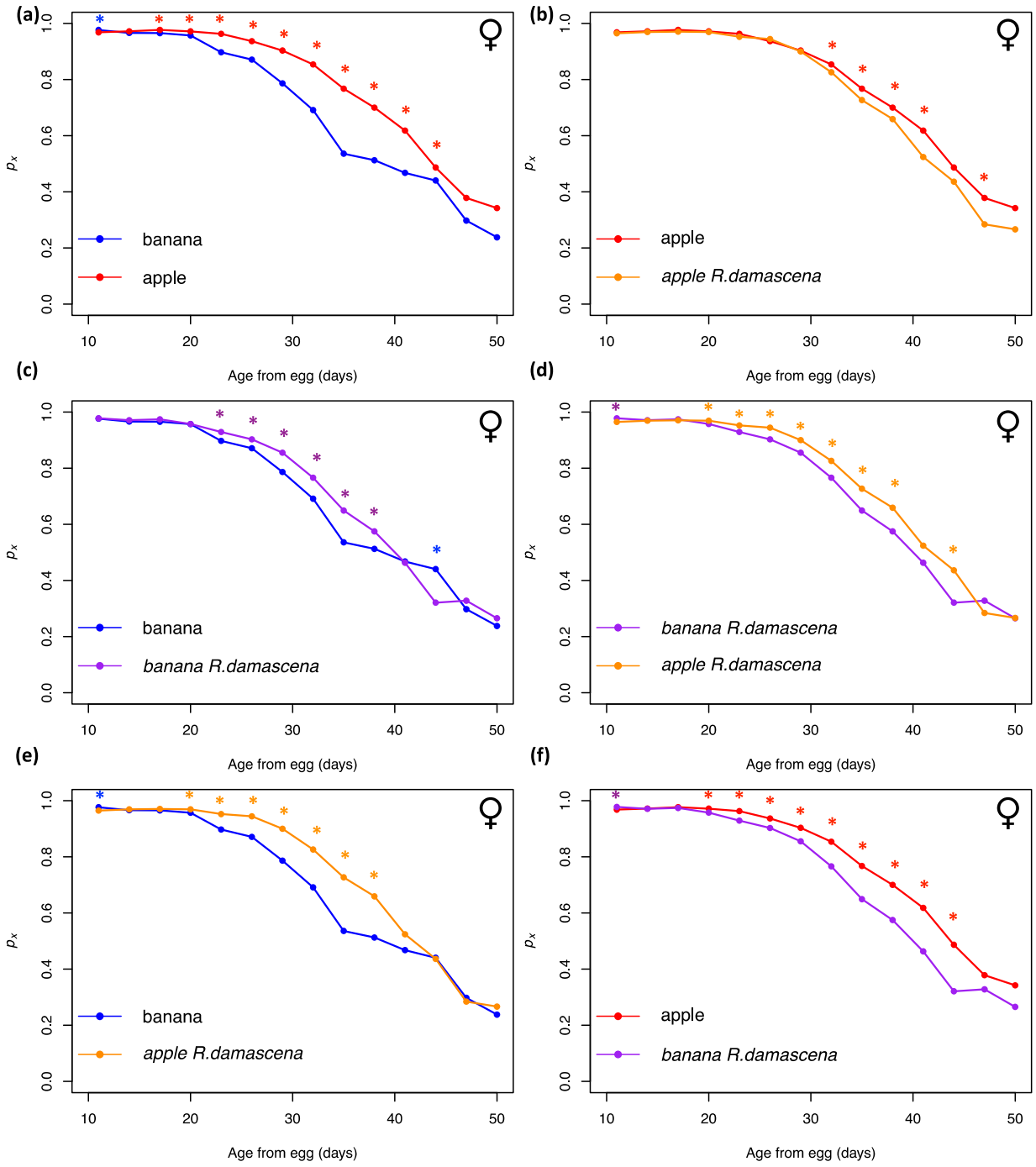
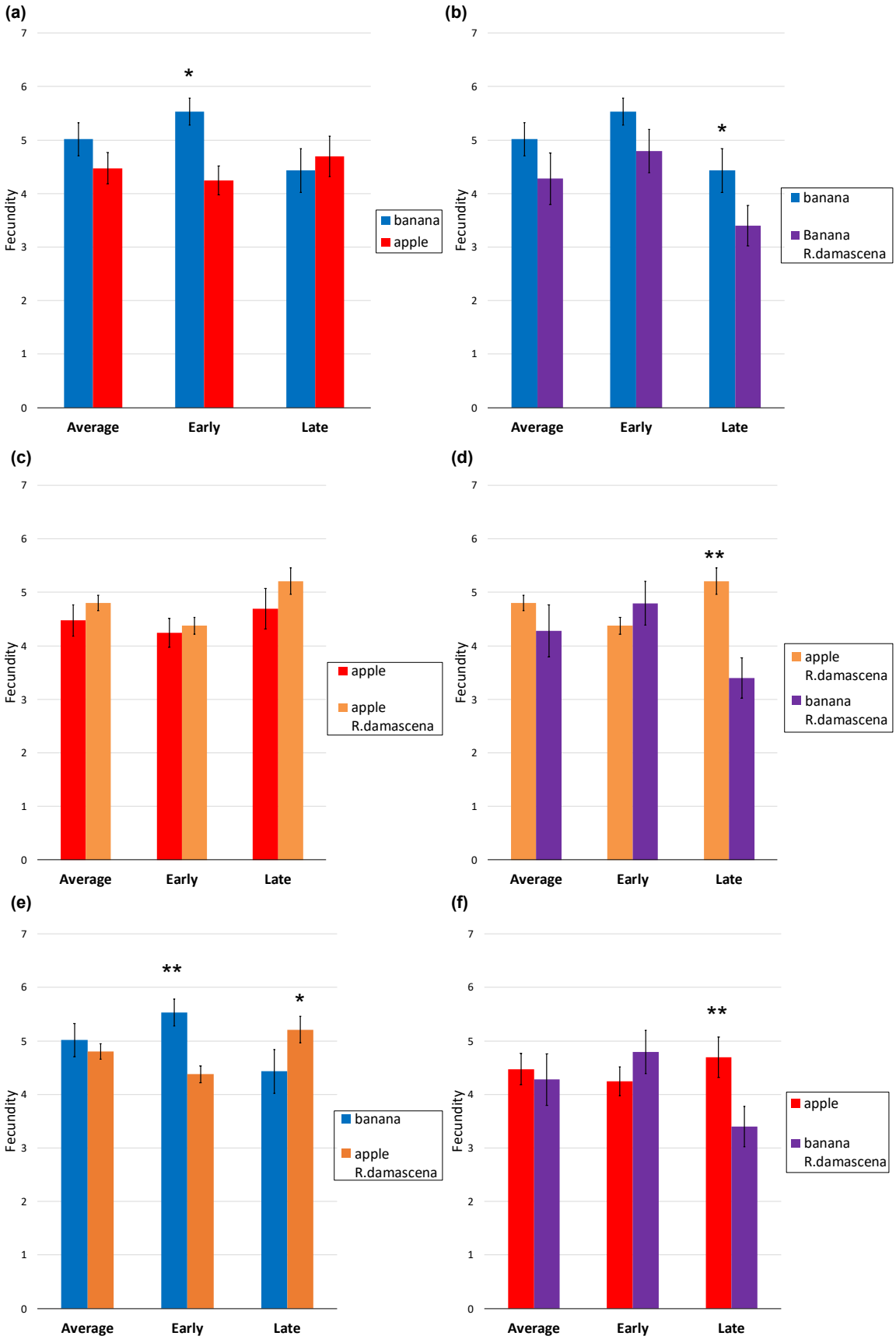


Figure 3.2 Conditional survival probability (p_x) over adult age (x) for females of the *Rosa damascena* (*R. damascena*) supplementation experiment. (a) banana vs. apple **(b)** apple vs. apple *Rosa damascena* **(c)** banana vs. banana *Rosa damascena* **(d)** banana *Rosa damascena* vs. apple *Rosa damascena* **(e)** banana vs. apple *Rosa damascena* **(f)** apple vs. banana *Rosa damascena*. Points represent p_x pooled across five replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk indicates which treatment has higher survivorship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apple
banana R.damascena

Figure 3.3 Average, early and late eggs per surviving female (m_x) across day 11-49, 11- 29, 30-49 days respectively from egg for the six different comparisons. (a) Early m_x is statistically higher in banana compared to apple (p=0.010). No difference in average or late m_x (p>0.05). **(b)** Late fecundity is statistically higher in the banana compared to the banana *Rosa damascena* (p=0.035). However, no difference is seen for average and early fecundity. **(c)** No difference in m_x is observed in apple compared to apple *Rosa damascena*. **(d)** Later m_x is statistically higher in the apple *Rosa damascena* compared to banana *Rosa damascena* (p=0.0003). **(e)** Earlier m_x is statistically higher in banana compared to apple *Rosa damascena* while later m_x is higher in the apple *Rosa damascena* (p=0.004 and p=0.045). **(f)** Later m_x is statistically higher in apple compared to banana *Rosa damascena* (p=.009). *p<0.05 **p<0.01. Errors bars show the mean ± 1 SEM

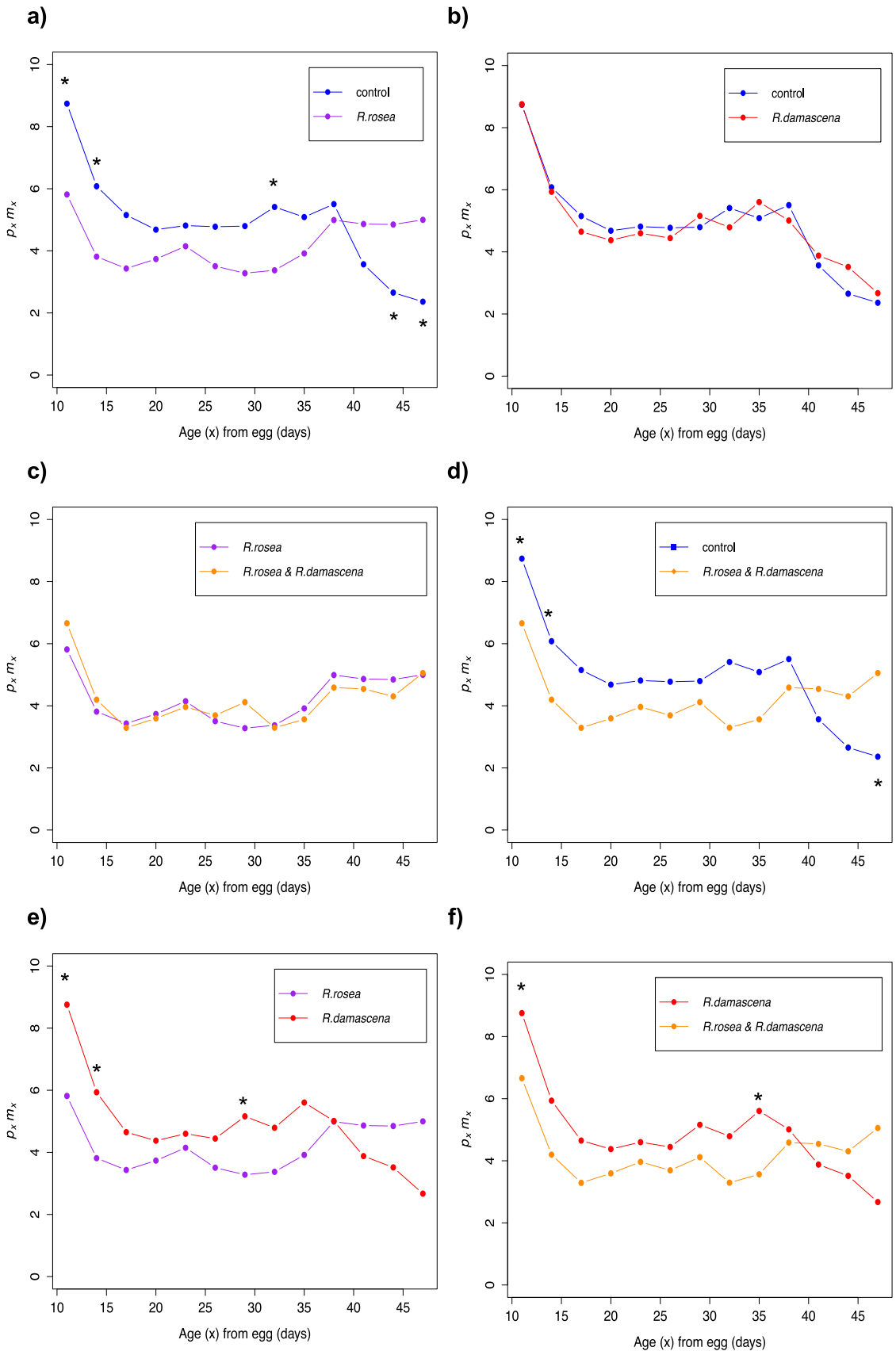
a)
b)
c)
d)
e)
f)
control
R.rosea
R.damascena
R.rosea & R.damascena
$p_x m_x$
Age (x) from egg (days)

Figure 3.4 Age-dependent fitness ($p_x m_x$) over adult age (x) for the *Rosa damascena* (*R. damascena*) and *Rhodiola rosea* (*R. rosea*) combination experiment. (a) The control treatment is generally higher at early ages and lower at later ages compared to the *R. rosea* treatment. **(b)** No difference is observed between the control and the *R. damascena* treatment at any age. **(c)** No difference is observed between the *Rhodiola rosea* and the combination treatment at any age. **(d)** The control treatment is generally higher at early ages and lower at later ages compared to the combination treatment. **(e)** The *Rosa damascena* treatment is generally higher at early ages and lower at later ages compared to the *Rhodiola rosea* treatment, however the later ages are not significant. **(f)** The *Rosa damascena* treatment is generally higher at early ages and lower at later ages compared to the combination treatment, however later ages are not statistically significant. Points represent the pooled data across five replicates averaged across three days. * denotes significance for that interval between shown diets ($p<0.05$)

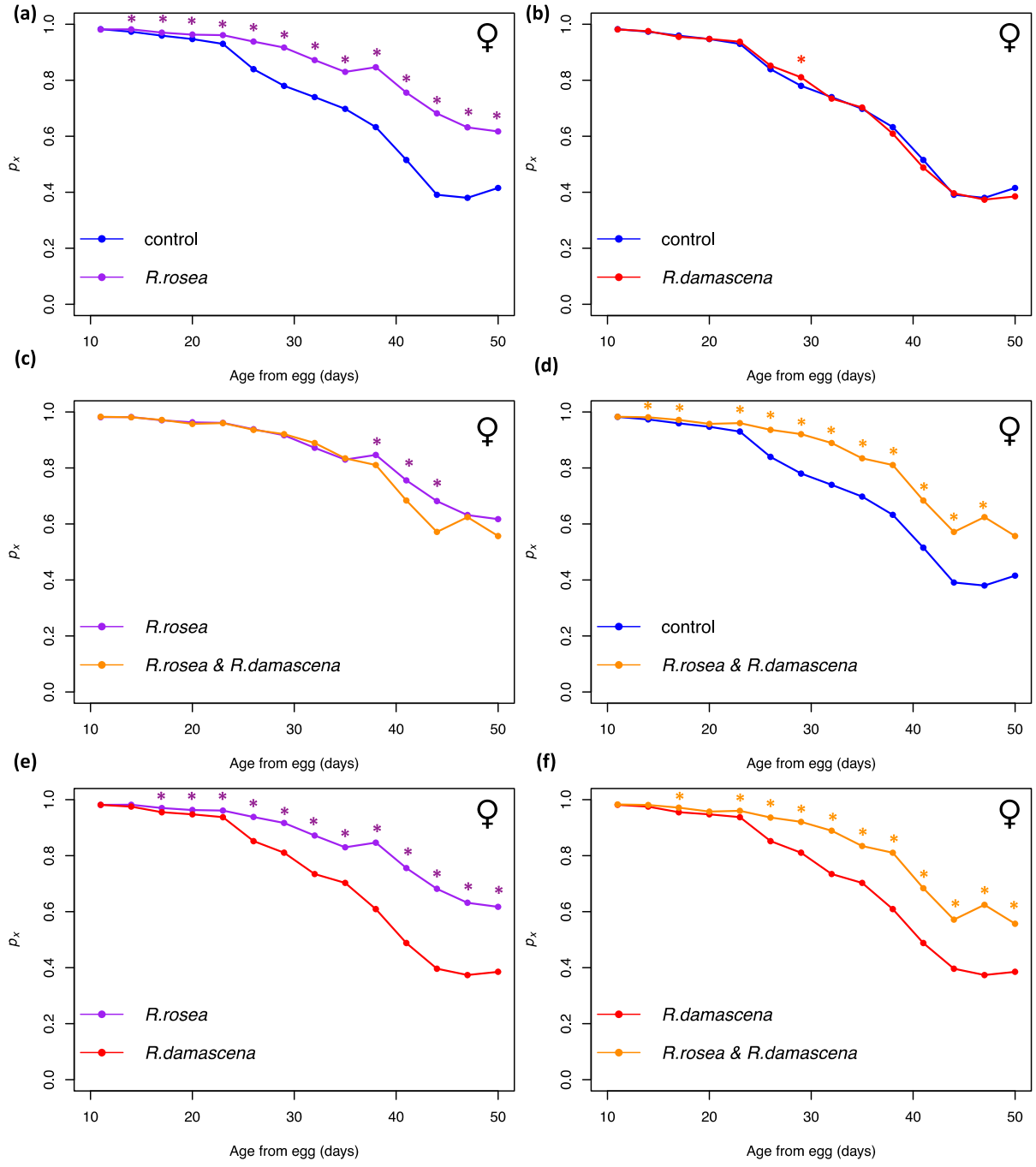

Figure 3.5 Conditional survival probability (p_x) over adult age from egg (x) for females of the *Rhodiola rosea* (*R. rosea*) & *Rosa damascena* (*R. damascena*) combination experiment. (a) control vs. *Rhodiola rosea* **(b)** control vs. *Rosa damascena* **(c)** *Rhodiola rosea* vs. combination **(d)** control vs. combination **(e)** *Rhodiola rosea* vs. *Rosa damascena* **(f)** *Rosa damascena* vs. combination. Points represent p_x pooled across five replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk indicates which treatment has higher survivorship

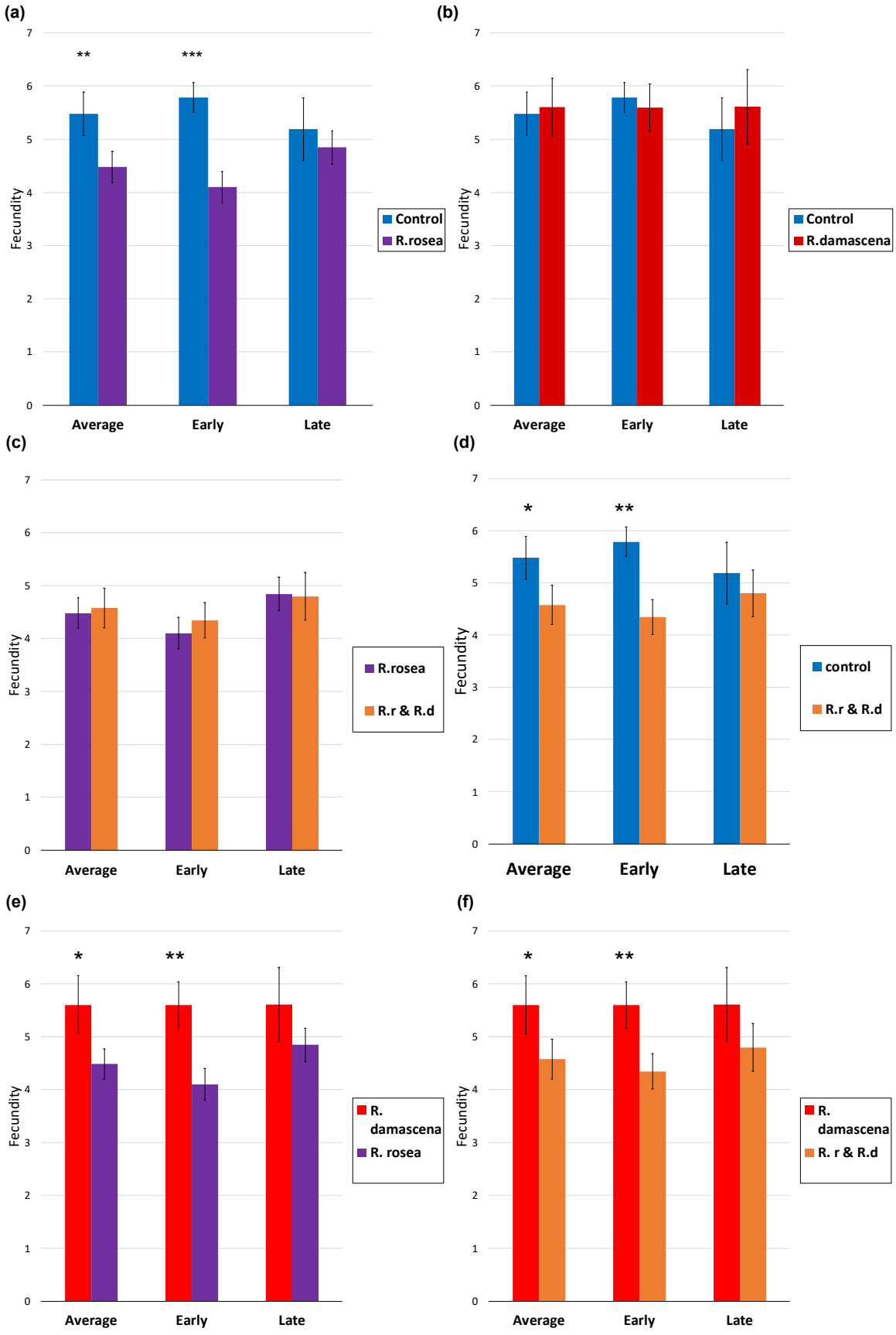
(a)
Fecundity
**
***
Average
Early
Late
Control
R.rosea
(b)
Fecundity
Average
Early
Late
Control
R.damascena
(c)
Fecundity
Average
Early
Late
R.rosea
R.r & R.d
(d)
Fecundity
*
**
Average
Early
Late
control
R.r & R.d
(e)
Fecundity
*
**
Average
Early
Late
R. damascena
R. rosea
(f)
Fecundity
*
**
Average
Early
Late
R. damascena
R. r & R.d

Figure 3.6 Average, early and late eggs per surviving female (m_x) across day 11-49, 11- 29, 30-49 days respectively from egg for the six different comparisons. (a) Average and earlier m_x is higher in the control compared to *Rhodiola rosea* (p=0.007 & p=0.00006). No difference in later m_x (p>0.05). **(b)** No difference in m_x is observed in the control compared to *Rosa damascena.* **(c)** No difference in m_x is observed in *Rhodiola rosea* compared to the combination. **(d)** Average and earlier m_x is higher in the control compared to the combination (p=0.02 & p=0.002). **(e)** Average and earlier m_x is higher in *Rosa damascena* compared to *Rhodiola rosea* (p=.04 & p=.005). **(f)** Average and earlier m_x is higher in *Rosa damascena* compared to the combination (p=.02 & p=.003). *p<0.05 **p<0.01 ***p<0.001. Error bars show the mean ± 1 SEM

TABLES

Table 3.1. Results from the mean longevity analysis for the *Rosa damascena* experiment. B=banana, A=apple, Rd = *Rosa damascena*. *p*-values less than 0.05 are bolded.

Comparison	Males		Females	
	% Difference in mean longevity	*p*-value	% Difference in mean longevity	*p*-value
BRd - B	+0.85	0.5889	+5.43	**0.0469**
A - B	+0.03	0.9435	+13.51	**<0.001**
ARd - B	- 1.71	0.2699	+10.15	**0.0003**
A - BRd	- 0.82	0.5408	+7.67	**0.0030**
ARd - BRd	- 2.54	0.1041	+4.48	0.0649
ARd - A	- 1.73	0.3005	- 2.96	0.2088

Table 3.2. Results from the mean longevity analysis for the *Rhodiola rosea* & *Rosa damascena* (combination) experiment. c=banana control, Rr = *Rhodiola rosea*, Rd = *Rosa damascena*, RrRd = combination. *p*-values less than 0.05 are bolded.

Comparison	Males		Females	
	% Difference in mean longevity	*p*-value	% Difference in mean longevity	*p*-value
Rr - c	+16.29	**<0.0001**	+18.83	**0.0003**
Rd - c	+1.40	0.6226	+0.75	0.8731
RrRd - c	+14.46	**<0.0001**	+17.09	**0.0008**
Rd - Rr	- 12.80	**<0.0001**	- 15.22	**0.0004**
RrRd - Rr	- 1.57	0.4686	- 1.46	0.6650
RrRd - Rd	+12.89	**0.0001**	+16.22	**0.0011**

SUPPLEMENTAL MATERIALS

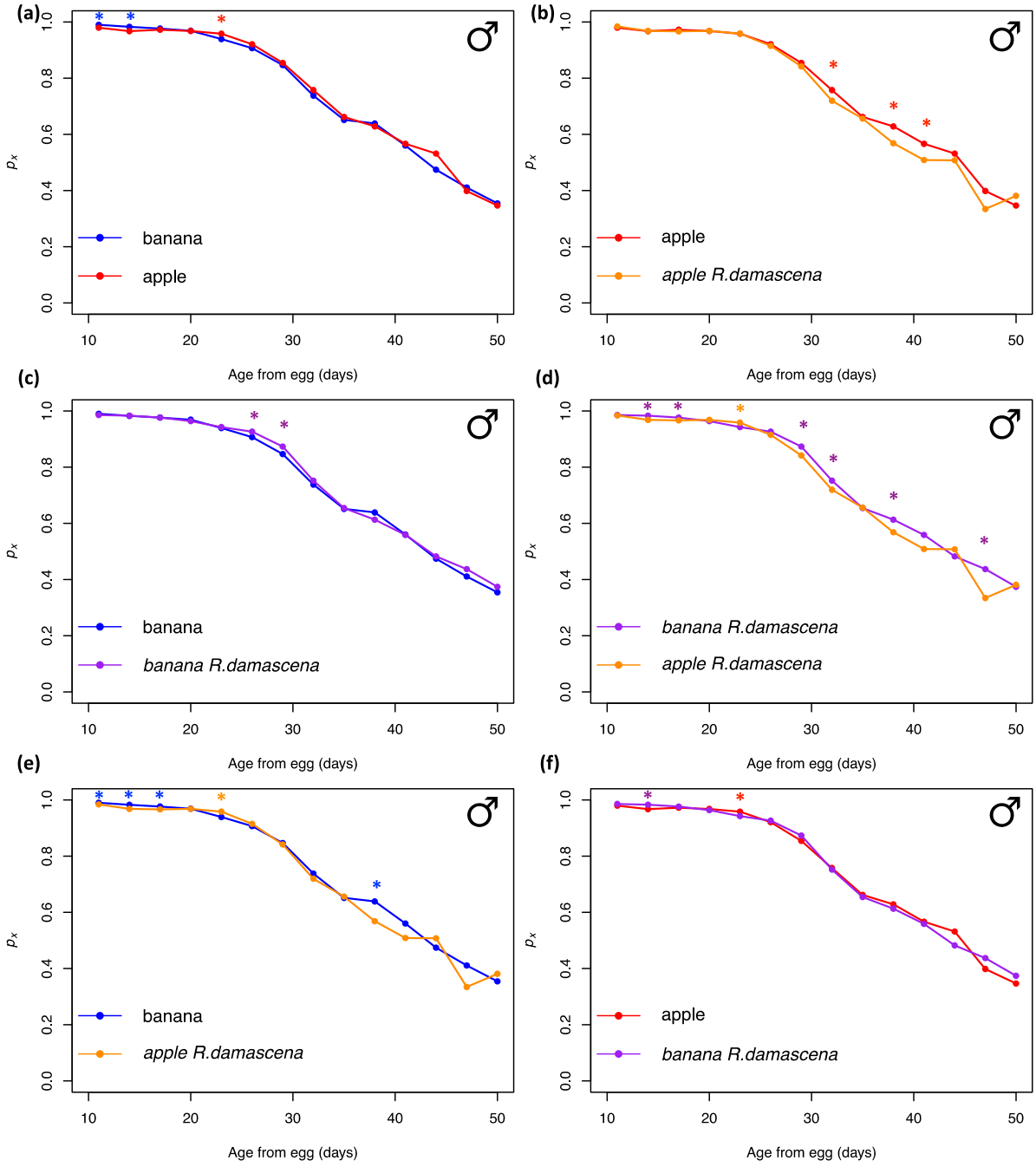

Figure S3.1 Conditional survival probability (p_x) over adult age (x) for males of the *Rosa damascena* (*R. damascena*) supplementation experiment. (a) banana vs. apple **(b)** apple vs. apple *Rosa damascena* **(c)** banana vs. banana *Rosa damascena* **(d)** banana *Rosa damascena* vs. apple *Rosa damascena* **(e)** banana vs. apple *Rosa damascena* **(f)** apple vs. banana *Rosa damascena*. Points represent p_x pooled across five replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk indicates which treatment has higher survivorship.

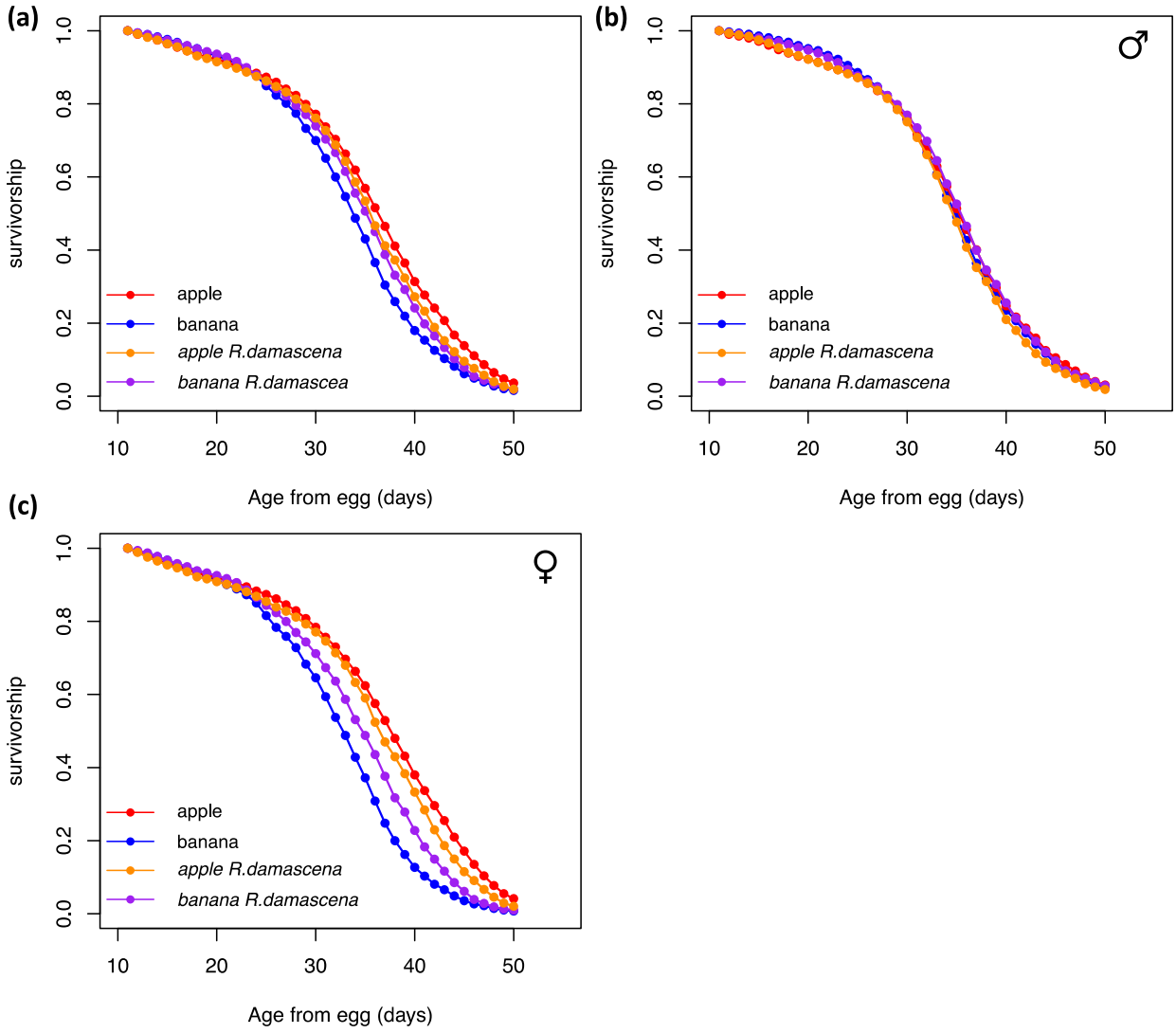

Figure S3.2 Survivorship for the apple, banana, apple *Rosa damascena*, and banana *Rosa damascena* treatments. (a) sexes pooled. **(b)** male flies. **(c)** female flies. Data is pooled across replicates

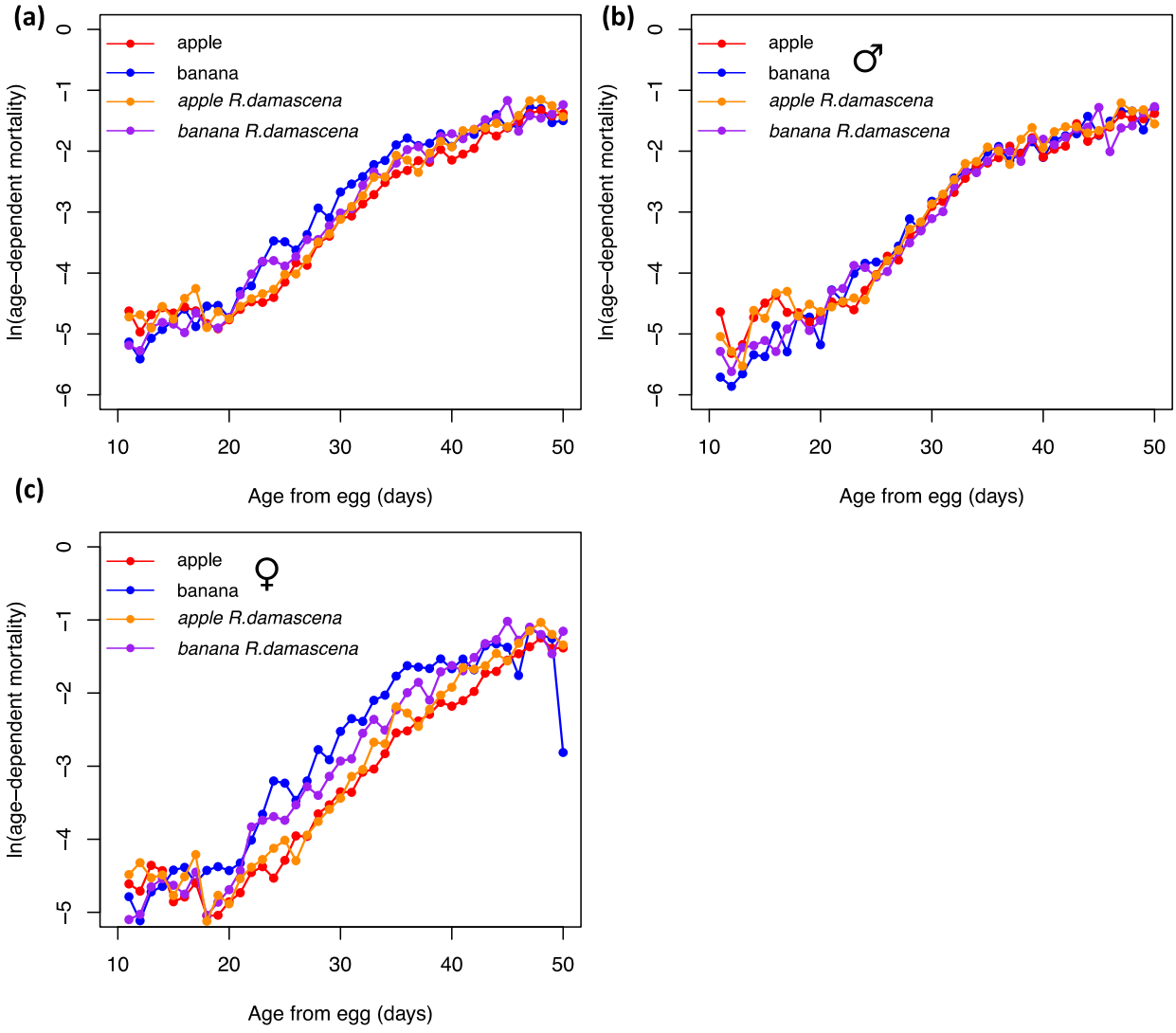

Figure S3.3 Natural log transformed age-dependent mortality for the apple, banana, apple *Rosa damascena*, and banana *Rosa damascena* treatments. (a) sexes pooled. **(b)** male flies. **(c)** female flies. Data is pooled across replicates

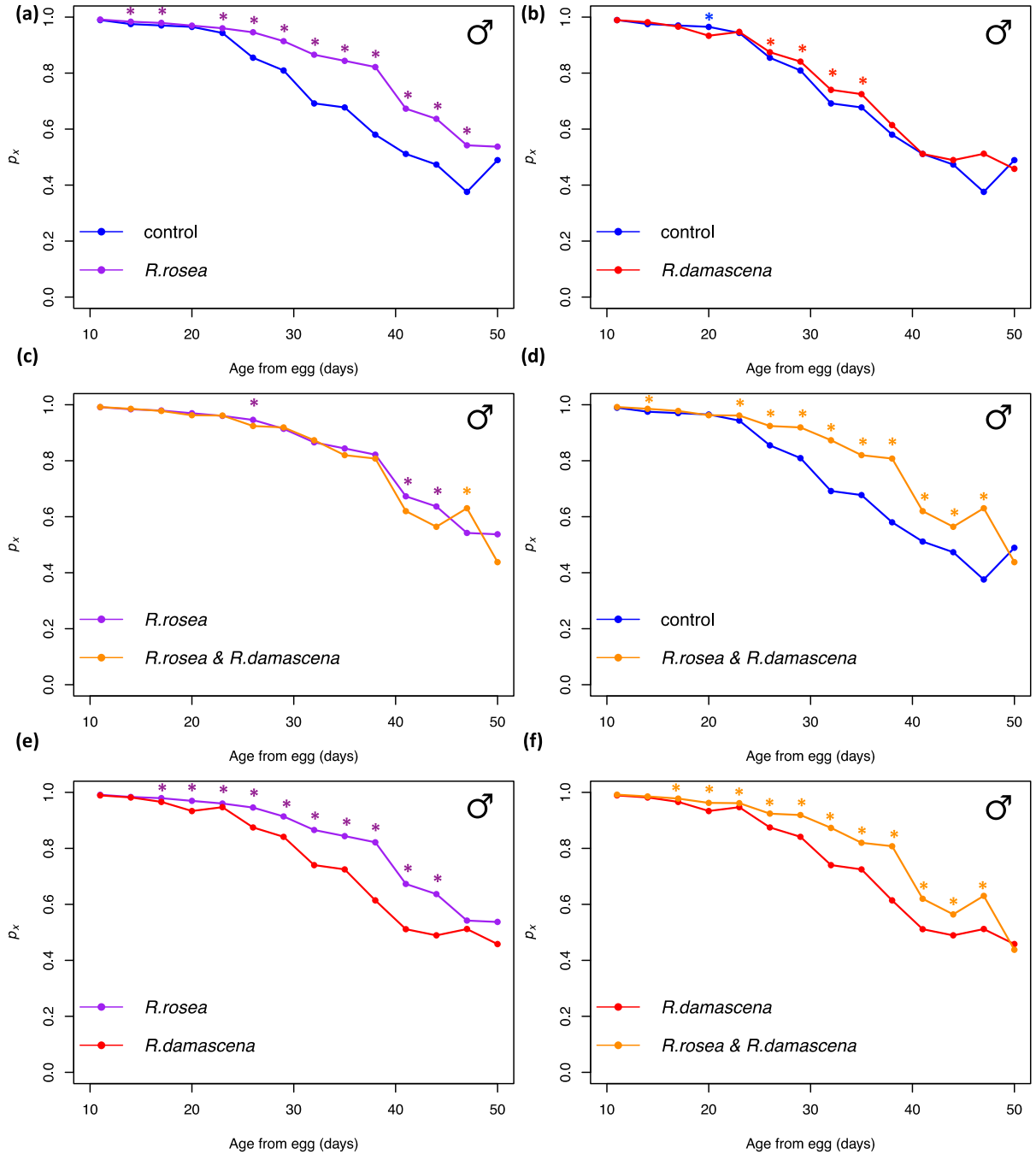

Figure S3.4 Conditional survival probability (p_x) over adult age from egg (x) for males of the *Rhodiola rosea* & *Rosa damascena* combination experiment. (a) control vs. *Rhodiola rosea* **(b)** control vs. *Rosa damascena* **(c)** *Rhodiola rosea* vs combination **(d)** control vs. combination **(e)** *Rhodiola rosea* vs. *Rosa damascena* **(f)** *Rosa damascena* vs. combination. Points represent p_x pooled across five replicates and pooled across three days. *denotes significance for that interval between shown diets (p<0.05). The color of the asterisk indicates which treatment has higher survivorship.

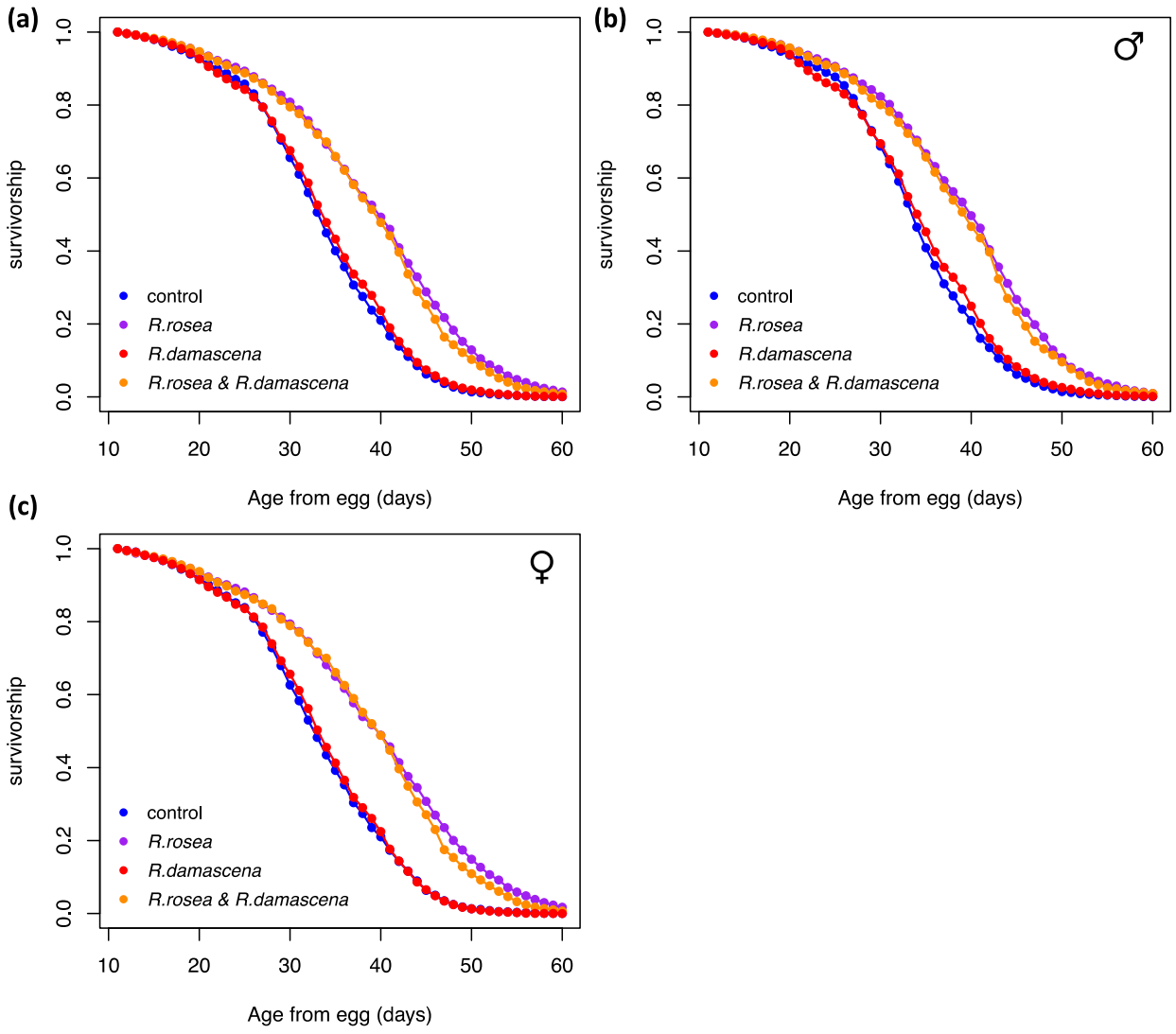

Figure S3.5 Survivorship for the control, *Rhodiola rosea*, *Rosa damascena*, and combination treatments. (a) sexes pooled. **(b)** male flies. **(c)** female flies. Data is pooled across replicates.

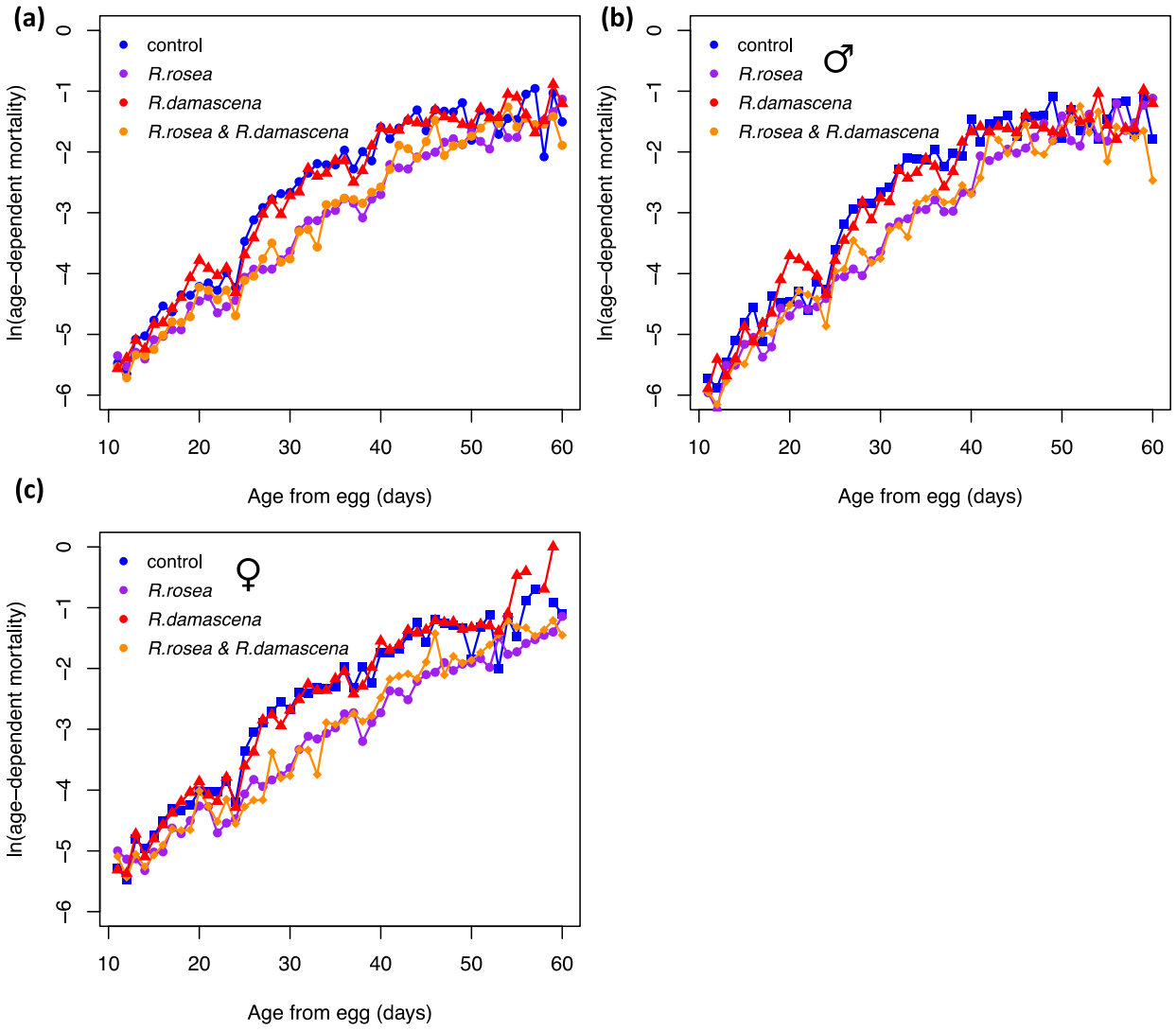

Figure S3.6 Natural log transformed age-dependent mortality for the control, *Rhodiola rosea*, *Rosa damascena*, and combination treatments. (a) sexes pooled. **(b)** male flies. **(c)** female flies. Data is pooled across replicates.

Table S3.1. Results from the interval regression analysis (lme) for the apple vs. banana flies. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (apple - banana)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-3.440	0.523	**<0.0001**	**<0.0001**
2	-1.781	0.523	**0.0036**	**0.017**
3	-0.794	0.523	0.15	0.45
4	-0.800	0.523	0.15	0.44
5	-0.987	0.523	0.077	0.27
6	0.224	0.523	0.67	0.97
7	-0.391	0.523	0.47	0.88
8	0.111	0.523	0.83	0.10
9	0.736	0.523	0.18	0.51
10	0.520	0.523	0.33	0.75
11	1.0958	0.529	0.054	0.20
12	1.735	0.546	**0.0058**	**0.027**
13	0.616	0.546	0.27	0.677

Table S3.2. Results from the interval regression analysis (lme) for the apple vs. apple *Rosa damascena*. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (apple–apple *R. damascena*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-0.543	0.523	0.31	0.73
2	-0.0768	0.523	0.89	1
3	0.00869	0.523	0.99	1
4	-0.452	0.523	0.40	0.82
5	-0.00839	0.523	0.99	1
6	-0.0229	0.523	0.97	1
7	0.391	0.523	0.47	0.88
8	-0.242	0.523	0.65	0.97
9	-0.0992	0.523	0.85	1
10	-1.0968	0.523	0.052	0.20
11	-0.486	0.523	0.37	0.79
12	-0.0564	0.523	0.92	1
13	-0.106	0.523	0.84	1

Table S3.3. Results from the interval regression analysis (lme) for the banana vs. banana *Rosa damascena*. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (banana– banana *R. damascena*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	1.373	0.523	**0.018**	0.078
2	1.562	0.523	**0.0087**	**0.039**
3	-0.0433	0.523	0.93	1
4	0.578	0.523	0.29	0.69
5	0.443	0.523	0.41	0.83
6	0.306	0.523	0.57	0.93
7	0.341	0.523	0.52	0.91
8	0.0695	0.523	0.90	1
9	-0.516	0.523	0.34	0.76
10	-0.00496	0.523	0.99	1
11	1.210	0.523	**0.036**	0.14
12	1.251	0.546	**0.036**	0.14
13	0.735	0.546	0.20	0.55

Table S3.4. Results from the interval regression analysis (lme) for the apple *Rosa damascena* vs. banana *Rosa damascena*. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (apple *R. damascena* – banana *R. damascena*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-1.524	0.523	**0.010**	**0.045**
2	-0.142	0.523	0.79	0.99
3	-0.846	0.523	0.13	0.40
4	0.230	0.523	0.67	0.97
5	-0.536	0.523	0.32	0.74
6	0.553	0.523	0.31	0.72
7	-0.441	0.523	0.41	0.83
8	0.422	0.523	0.43	0.85
9	0.319	0.523	0.55	0.93
10	1.612	0.523	**0.0071**	**0.032**
11	2.791	0.523	**0.00010**	**0.00030**
12	3.0424	0.523	**<0.00010**	**0.00010**
13	1.457	0.523	**0.013**	0.058

Table S3.5. Results from the interval regression analysis (lme) for the apple *Rosa damascena* vs. banana. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (apple *R. damascena*- banana)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-2.897	0.523	**<0.00010**	**0.00020**
2	-1.704	0.523	**0.0049**	**0.023**
3	-0.802	0.523	0.14	0.44
4	-0.348	0.523	0.52	0.91
5	-0.979	0.523	0.079	0.28
6	0.247	0.523	0.64	0.96
7	-0.782	0.523	0.15	0.46
8	0.353	0.523	0.51	0.91
9	0.835	0.523	0.13	0.41
10	1.617	0.523	**0.0070**	**0.032**
11	1.581	0.523	**0.0087**	**0.039**
12	1.791	0.523	**0.0047**	**0.022**
13	0.722	0.523	0.20	0.56

Table S3.6. Results from the interval regression analysis (lme) for the apple vs. banana *Rosa damascena*. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (apple - banana *R. damascena*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-2.0666	0.523	**0.0011**	**0.0056**
2	-0.219	0.523	0.68	0.97
3	-0.837	0.523	0.13	0.41
4	-0.222	0.523	0.68	0.97
5	-0.544	0.523	0.31	0.73
6	0.530	0.523	0.33	0.74
7	-0.0500	0.523	0.92	1
8	0.180	0.523	0.73	0.99
9	0.220	0.523	0.68	0.97
10	0.515	0.523	0.34	0.76
11	2.305	0.523	**0.00040**	**0.0022**
12	2.986	0.523	**<0.00010**	**0.00020**
13	1.351	0.523	**0.020**	0.084

Table S3.7. Results from the interval regression analysis (lme) for the control vs. *Rhodiola rosea* flies. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (control - *R. rosea*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	2.898	0.643	**0.00040**	**0.0018**
2	2.234	0.643	**0.0031**	**0.015**
3	1.695	0.643	**0.018**	0.076
4	0.936	0.643	0.16	0.48
5	0.655	0.643	0.32	0.74
6	1.261	0.643	0.068	0.24
7	1.513	0.643	**0.032**	0.13
8	2.0879	0.643	**0.0050**	**0.023**
9	1.471	0.643	**0.036**	0.14
10	0.908	0.643	0.18	0.51
11	-1.523	0.643	**0.031**	0.12
12	-2.126	0.643	**0.0045**	**0.021**
13	-1.957	0.643	**0.0077**	**0.035**

Table S3.8. Results from the interval regression analysis (lme) for the control vs. *Rosa damascena* flies. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (control - *R. damascena*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-0.0704	0.643	0.91	1
2	0.112	0.643	0.86	1
3	0.471	0.643	0.47	0.88
4	0.278	0.643	0.67	0.97
5	0.179	0.643	0.78	0.99
6	0.305	0.643	0.64	0.96
7	-0.394	0.643	0.55	0.93
8	0.660	0.643	0.32	0.74
9	-0.348	0.643	0.60	0.95
10	0.842	0.643	0.21	0.57
11	-0.523	0.643	0.43	0.85
12	-1.177	0.643	0.086	0.30
13	-0.785	0.643	0.24	0.62

Table S3.9. Results from the interval regression analysis (lme) for the combination vs. *Rhodiola rosea* flies. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (combination-*R. rosea*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	0.830	0.643	0.21	0.58
2	0.390	0.643	0.55	0.93
3	-0.134	0.643	0.84	1
4	-0.126	0.643	0.85	1
5	-0.159	0.643	0.81	0.99
6	0.211	0.643	0.75	0.99
7	0.844	0.643	0.21	0.57
8	-0.0740	0.643	0.91	1
9	-0.290	0.643	0.66	0.97
10	-0.240	0.643	0.71	0.98
11	-0.326	0.643	0.62	0.96
12	-0.466	0.643	0.48	0.89
13	0.225	0.643	0.73	0.98

Table S3.10. Results from the interval regression analysis (lme) for the combination vs. control flies. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-Interval	**Difference (combination-control)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-2.0671	0.643	**0.0054**	**0.025**
2	-1.843	0.643	**0.011**	**0.049**
3	-1.829	0.643	**0.012**	0.052
4	-1.0621	0.643	0.12	0.38
5	-0.814	0.643	0.22	0.60
6	-1.0494	0.643	0.12	0.39
7	-0.669	0.643	0.31	0.73
8	-2.162	0.643	**0.0040**	0.019
9	-1.761	0.643	**0.015**	0.063
10	-1.147	0.643	0.093	0.32
11	1.197	0.643	0.081	0.28
12	1.660	0.643	**0.020**	0.085
13	2.183	0.643	0.0037	**0.018**

Table S3.11. Results from the interval regression analysis (lme) for the *Rhodiola rosea* vs. *Rosa damascena*. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (*R. rosea* - *R. damascena*)**	**se**	***p*-value**	***p*-value (Tukey)**
1	-2.968	0.643	**0.00030**	**0.0015**
2	-2.122	0.643	**0.0045**	**0.021**
3	-1.224	0.643	0.075	0.27
4	-0.658	0.643	0.321	0.74
5	-0.476	0.643	0.47	0.88
6	-0.956	0.643	0.16	0.47
7	-1.907	0.643	**0.0091**	**0.041**
8	-1.428	0.643	**0.041**	0.16
9	-1.820	0.643	**0.012**	0.053
10	-0.0657	0.643	0.92	01
11	1.000	0.643	0.14	0.43
12	0.949	0.643	0.16	0.47
13	1.173	0.643	0.087	0.30

Table S3.12. Results from the interval regression analysis (lme) for the combination vs. *Rosa damascena*. Significant *p*-values are bolded. se = standard error for the difference. "Tukey" includes adjusted *p*-values so that the type-I error for tests on all comparisons is 0.05.

Age-interval	**Difference (combination – *R. damascena*)**	**SE**	***p*-value**	***p*-value (Tukey)**
1	-2.138	0.643	**0.0043**	**0.020**
2	-1.731	0.643	**0.016**	0.069
3	-1.358	0.643	0.051	0.19
4	-0.784	0.643	0.24	0.62
5	-0.635	0.643	0.34	0.76
6	-0.745	0.643	0.26	0.66
7	-1.0633	0.643	0.12	0.38
8	-1.502	0.643	**0.033**	0.13
9	-2.110	0.643	**0.0047**	**0.022**
10	-0.305	0.643	0.64	0.96
11	0.674	0.643	0.31	0.72
12	0.483	0.643	0.46	0.88
13	1.398	0.643	**0.045**	0.17

CHAPTER 4

Effects of evolutionary history on phenotypic convergence in *Drosophila melanogaster* populations selected for extreme desiccation resistance and larval urea tolerance

ABSTRACT

Studies in the field of experimental evolution have found that the response to selection is rapid and highly repeatable across sexually reproducing replicate populations. In addition, convergence upon ancestral phenotypes in differentiated populations occurs quickly, sometimes within only a few dozen generations. However, other experiments have shown that populations subjected to reverse selection may respond quickly, slowly, or not at all depending on the character studied and past evolutionary history. Here we assayed *D. melanogaster* populations that were selected for intense desiccation resistance for approximately 250 generations and have since been relaxed for an additional 250 generations. We monitored a second set of populations that have long been selected for larval tolerance to toxic levels of urea and an additional set that have been undergoing relaxed selection for ~80 generations. We found that the previously selected desiccation populations live approximately seven days longer than their controls after 250 generations of relaxed selection, a similar difference to what they exhibited at their peak of differentiation for this character. The urea selected populations and their relaxed counterparts show no difference in larva to adult viability and adult mean lifespan after larval exposure to urea. These findings suggest that extreme selection has long-lasting impacts on phenotypic differentiation for one character, longevity. Relaxed selection in the urea selected populations will continue to be monitored for signs of phenotypic convergence.

INTRODUCTION

Studies performed in outbred populations of *Drosophila* demonstrate that laboratory evolution can be rapid and highly repeatable (Matos et al. 2000; Simões et al. 2008; Archer et al. 2003; Burke et al. 2016). When populations of *Drosophila melanogaster* are selected for different patterns of reproduction, phenotypic divergence from ancestral populations occurs within dozens of generations for characters such as longevity, development time, and fecundity (Burke et al. 2016). In addition, populations sharing a selection regime converge in a similar timeframe irrespective of past selection pressures (Burke et al. 2016). Studies with populations of *Drosophila suboscura* collected from different European latitudes likewise show that initial differentiation is quickly reduced when populations are raised in common laboratory environments (Fragata et al. 2014).

Cumulatively, these findings suggest that phenotypes are primarily shaped by the most recent selection regimes imposed on a population, and that evolutionary history has little impact. However, reverse experimental evolution studies sometimes suggest that reversal back to the ancestral state depends on evolutionary history and the character studied (Teotónio and Rose 2000; Passananti et al. 2004). Teotónio and Rose (2000) show that in 25 diverged populations of *D. melanogaster*, the patterns of reverse evolution varied among selection histories and the characters studied. For example, some characters respond quickly with full convergence upon the ancestral values within 20 generations. Others show a response but fail to converge after 50 generations. In the same vein, Passananti et al. (2004) relaxed selection for ~40 generations on stocks selected for resistance to either starvation or desiccation. They found that relaxed selection did not significantly change longevity, early fecundity, and desiccation resistance, characters all differentiated in the long-standing populations. However, these studies only monitored reverse selection for

approximately 50 generations or less. It is unclear whether complete convergence would occur if given more time. In fact, Service et al. (1988) found that after 20 generations, response to reverse selection for some characters was negligible, but after more than 100 generations in the ancestral environment, convergence occurred.

Teotonio et al. (2000) did not find that the lack of genetic variation was a reason for the failure of some characters to return to the ancestral state. Instead they proposed that, "the return to the ancestral environment did not produce uniform selection pressures among populations of different evolutionary histories" (Teotonio and Rose 2000). In other words, the lack of complete convergence could be due to differential genotype-by environment interaction (Teotonio and Rose 2001).

Here we test whether evolutionary history matters in populations where past generations were subjected to intense selective pressures and have since been relaxed for ~250 generations. We test this hypothesis using a group of *D. melanogaster* populations that were subjected to intense selection for desiccation resistance. In addition, we monitored the effect of reverse selection in populations that have been subjected to selection for increased larval tolerance to toxic levels of urea.

We start by examining patterns of phenotypic differentiation in two five-fold replicated stocks, TSO $_{1\text{-}5}$, and TDO $_{1\text{-}5}$, known as C and D respectively during active selection, which were first described in Rose *et al*. (1992). The D populations were intensely selected for desiccation resistance for about 260 generations, and afterward were renamed as TDO, and maintained on a 21 day (T for "Three-week") relaxed culture selection over the past ~230 generations. The C populations were moderately selected for starvation resistance for about 260 generations in parallel with the D populations, serving as controls for the D populations, and were later renamed as TSO,

and maintained under the same culture selection regime as the TDO populations. The TSO and TDO populations were all placed under relaxed culture selection during the same generations. The extreme differentiation of such characters as carbohydrate content, water loss rates, and water content initially differentiating these two groups of (C, D) populations was achieved using environments so inimical to survival that only a small percentage (10-20%) of each generation survived selection (Rose et al. 1992; Gibbs et al. 1997; Djawdan et al. 1998; Archer et al. 2003). We have called this intense selection paradigm "culling selection" in the past, and it represents one of the most extreme protocols used in Drosophila experimental evolution (Rose et al. 1990).

We will end by examining phenotypic differentiation in three five-fold replicated stocks, AUC_{1-5}, UX_{1-5}, and RUX_{1-5}. The AUC and UX populations were first described in Borash et al. (2000). AUC_{1-5} and UX_{1-5} were derived from UU_{1-5} (Joshi and Mueller 1996a). The UX populations were selected for larval tolerance of urea for ~350 generations. The AUC populations were maintained identically to the UU populations and served as controls for the UX populations. The RUX_{1-5} were derived from the UX_{1-5} and are maintained identically to the AUC populations. The RUX populations have undergone ~80 generations of relaxed selection.

MATERIALS AND METHODS

Populations

These experiments used large, outbred lab populations of *Drosophila melanogaster* derived from a population sampled by P.T. Ives from South Amherst, Massachusetts (Ives, 1970).

The first set of experimental stocks used in this study were derived from a set of 5 populations that had been selected for late reproduction (O_{1-5}). The O_{1-5} populations were derived from the Ives stock in February 1980 (Rose 1984). In 1988, two sets of populations were derived from the O_{1-5} populations. One set (D_{1-5}) were selected for desiccation resistance while the other set (C_{1-5}) were maintained to control for desiccation resistance selection. The C_{1-5} populations were handled like the D_{1-5} populations, except flies were given nonnutritive agar instead of desiccant (Rose et al. 1992). In 2005, these populations were relaxed from selection and kept on a 21-day culture regime that is sustained to the present day. Under this new regime, the D populations have been renamed "TDO," and the C populations "TSO." In total, the TDO populations underwent ~260 generations of selection for desiccation resistance, and ~230 generations of relaxed selection.

The second set of experimental stocks used in this study (Figure S5.1) were derived from five UU populations (Joshi and Mueller, 1996a). The UU populations were derived in the Fall of 1991 from the five B populations of Rose (1984) and were maintained on a 3-week generation cycle. In Fall 1996, five UX and five AUC populations were derived from the UU populations. The UX populations were maintained in the same fashion as the UU populations, except that urea was added to the standard banana-molasses food. These populations were exposed to urea during their vial phase only (day 0-14). The concentration of urea started at 12g/L banana food and was ramped up to 18g/L at generation 20 (Borash et al. 2000). It has since been reduced to 16g/L, and

the culture protocol has remained at this concentration for hundreds of generations. The five AUC populations served as controls for the UX and were maintained with the same life cycle and culture environment, but without the added urea. In the Winter of 2013, five RUX populations were derived from the five UX populations. The RUX populations are maintained in the same manner as the AUC populations. The UU populations are now extinct.

Populations were reared on a banana-molasses diet for stock maintenance and for experimental assays. The banana-molasses media is composed of the following ingredients per 1L distilled H_20: 13.5g Apex® Drosophila agar type II, 121g peeled, ripe banana, 10.8mL light Karo® corn syrup, 10.8mL dark Karo® corn syrup, 16.1mL Eden®organic barley malt syrup, 32.3g Red Star® active dry yeast, 2.1g Sigma-Aldrich® Methyl 4-hydroxybenzoate (anti-fungal), and 42.5 mL EtOH. Stocks are maintained on a 24-hour light cycle and kept at room temperature (24ºC ± 1ºC).

Phenotypic Assays for TSO and TDO

Mortality and Mean Longevity

For this assay, the TDO and TSO populations were reared in 8-dram polystyrene vials with ~6mL of food, an egg density of 60-80 eggs and given 14 days to develop. Adult flies from each replicate were transferred on day 14 from egg to three, six-liter acrylic plastic cages with ~1000 flies per cage (~3,000 flies per replicate). Flies were given fresh food daily, and every two weeks flies were transferred to clean cages using light CO_2 anesthesia. Individual mortality was assessed every 24 hours, the flies were sexed at death, and the exact cohort size was calculated from the complete recorded deaths. Total cohort size across all replicates from both regimes was ~30,000 flies.

Mean longevity was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The model used for the data is described as follows: Let y_{ijkm} be the longevity for regime – i (i =1 (TDO) or 2 (TSO)), sex-j (j=1 (female), 2(male)), population – k (k=1,.., 10) and individual – m (k=1,.., n_{jk}). A LME model for longevity is,

$$y_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where $\delta_s = 0$, if $s = 1$, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively.

Mortality rates from the TDO and TSO populations were analyzed using a two-stage, three-parameter Gompertz model. The Gompertz model and its variants describe the change in instantaneous mortality rates with age. The chance of dying between day t and t+1, q_t, was estimated as, $q_t = 1 - \frac{p_{t+1}}{p_t}$ where

$$p_t = \begin{cases} \exp\left\{\dfrac{A[1-\exp(\alpha t)]}{\alpha}\right\} \; if \; t \le bd \\ exp\left\{\dfrac{A[1-\exp(\alpha bd)]}{\alpha} + Aexp(\alpha bd)(bd-t)\right\} if \; t > bd \end{cases}$$

where bd is the break day or the age at which mortality rates transition from a Gompertz dynamic to a plateau.

With this model we let y_{ijkt} be the mortality from selection regime-i (i=1 (TDO), 2 (TSO)), sex-j (j=1 (female), 2(male)) and population-k (k=1, 2, …, 10), at age-t. Random variation arises due to both population effects and individual variation. Consequently, the mortality of adults from

selection regime-i, sex-j, and population-k, at time-t is $y_{ijkt} = f(\square_{ijk}, t) + \square_{ijkt}$, where $\square_{ijk}$ is the vector of parameters, (A_{ijk}, $\square_{ijk}$, bd_{ijk}), and,

$$A_{ijk} = \pi_1 + \delta_i\beta_{1i} + \delta_j\gamma_1 + b_{1k}$$

$$\alpha_{ijk} = \pi_2 + \delta_i\beta_{2i} + \delta_j\gamma_2 + b_{2k}$$

$$bd_{ijk} = \pi_3 + \delta_i\beta_{3i} + \delta_j\gamma_3 + b_{3k}$$

where $\square_s$ =0, if s=1 and 1 otherwise. The within population variation, $\square$, is assumed to be normally distributed with a zero mean. This variation increases with age so we assumed that Var($\square$)= $\sigma^2|t|^{2\Delta}$ where $\square$ is estimated from the data. Population variation, b_{mk}, was assumed to affect all three parameters. We tested models with population variation in subsets of parameters and with a constant within population variation. The model chosen had the lowest Akaike and Bayesian information criterion (Pinheiro 2015). The population variation is assumed independent of the within population variation and also has a normal distribution with zero mean and covariance matrix, $\mathbf{\Sigma}_b$. Parameters of equation (Z) were estimated by the restricted maximum likelihood techniques implemented by the *nlme* function in R.

Development Time: Larvae to Adult

In this experiment, the time from larvae hatching from egg to adult eclosion from pupae was studied. Eggs from the TDO and TSO populations were collected on non-nutritive agar. From each agar plate, 50 first-instar larvae were transferred to polystyrene vials with banana molasses food. 13 vials per replicate were assayed. Vials were checked every six hours after the first adult flies eclosed, and all eclosed flies were counted and sexed by microscope.

Time to eclosion was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The model used for the data is described as follows: Let y_{ijkm} be the development time for regime – i (i =1 (TDO) or 2 (TSO)), sex-j (j=1

(female), 2(male), population – k (k=1,.., 10) and individual – m (m=1,.., n_{jk}). A LME model for time to eclosion is,

$$y_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where $\delta_s = 0$, if $s = 1$, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively.

Adult Age-specific Fecundity

TDO and TSO adult age-specific fecundity was monitored for two weeks. Populations were reared in vials and given 14 days to develop. On day 14 from egg, one mating pair (one male and one female) were transferred to 60 charcoal caps per replicate. Charcoal medium is composed of the following per 1L distilled H_2O: 19g Apex® Drosophila agar type II, 5g Fisher® Activated Darco® G-60 Carbon, 54g Sucrose, 32g Red Star® active dry yeast, 3g Sigma-Aldrich® Methyl 4-hydroxybenzoate (anti-fungal), and 30mL EtOH. Starting on day 14, fecundity was monitored every 24 hours until day 28. Pairs were given a fresh charcoal cap with 50 µL yeast solution (98mL distilled water, 2g active dry yeast, and 2mL 1% acetic acid) each day, and the old charcoal caps were scanned on a flatbed scanner and counted at a later time.

Age-specific fecundity was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The data consisted of fecundity at an age (x) within an age interval - k (k = 1..,5). Fecundity was modeled by a straight line within each interval. Regime - j (j =1 (TDO) or 2 (TSO)) could affect the intercept, but not the slope of the line. Slope could vary between intervals. Populations - i (i = 1, 2…,10) contributed random variation to these measures. Fecundity at age (x), interval (k), regime (j), and population (i) is y_{ijkx} and can be described by,

$$y_{ijkx} = \alpha + \beta_k + \delta_j\gamma_j + (\omega + \pi_k\delta_k)x + \delta_k\delta_j\mu_{jk} + c_i + \mathcal{E}_{ijkx},$$

where $\delta_s = 0$ if $s = 1$ and 1 otherwise, and c_i and ε_{ijkx} are independent standard normal random variables with variance σ_c^2 and σ_ε^2, respectively. The effects of diet on the intercept are assessed by considering the magnitude and variance of both γ_j and μ_{jk}.

Fungal-resistance

Susceptibility to fungal infection was compared between the TDO and TSO populations. The pathogen used was the entomopathogenic fungus *Beauveria bassiana*, strain 12460 obtained from the USDA Agricultural Research Service Collection of Entomopathogenic Fungi, Ithaca NY. Fungal suspensions were prepared by suspending 0.3g of *B. bassiana* spores in 25mL of 0.03% silwet. The TDO and TSO populations were reared in vials and given 12 days to develop. On day 12, the flies were transferred to fresh food vials. On day 14, ~500 flies (sexes mixed) were briefly anesthetized with CO_2 and then placed on Petri Dishes on ice for the duration of the inoculation assay (<2 minutes). Anesthetized flies were sprayed either with 5mL of the prepared fungal suspension or with 5mL of control suspension (0.03% silwet, but not fungus) using a spray tower (Vandenberg 1996). Sprayed flies were then moved to 3L cages and kept at 100% humidity for 24 hours. After 24 hours, the humidity was reduced to 60%. Dead flies were removed from the cages daily and were sexed. Food was replaced daily. We completed three technical replicates and tested a total of ~1500 flies (sexes mixed) per population per treatment.

Fly mortality, $p_{ij}(t)$, was modeled at day-t (t= 1, 2,..,?) in selection regime-i (i=1 (TDO), 2 (TSO)) and treatment-j (j=1 (fungus), 2 (no fungus)) by the logistic regression function,

$$log\left[\frac{p_{ij}(t)}{1-p_{ij}(t)}\right] = \mu_0 + \delta_i\alpha_0 + \delta_j\beta_0 + \delta_i\delta_j\gamma_0 + \left(\mu_1 + \delta_i\alpha_1 + \delta_j\beta_1 + \delta_i\delta_j\gamma_1\right)t,$$

where □$_{k\ k}$=1 if k=1 or 0 otherwise. Parameters of this equation were estimated with the *glm* function in R (https://www.r-project.org).

Phenotypic Assays for AUC, UX, RUX

Mean Longevity after larvae urea exposure

At generation 0, 14, and 31 of RUX relaxed selection, the five AUC, UX, and RUX populations were assayed to determine adult mean longevity after exposure to urea as larvae. Prior to the experimental collect, all 15 populations were maintained on banana food for two generations. Populations were then reared in 8-dram polystyrene vials with either ~6mL of banana food or urea food (16g/L food), at an egg density of 60-80 eggs, and given 14 days to develop. ~1000 adult flies from each replicate treatment were transferred with light CO2 anesthesia on day 14 (from egg) to one, six-liter acrylic plastic cage (30 cages total). Every two weeks, flies were transferred to clean cages using light CO_2 anesthesia. Cages were feed fresh banana food daily (without urea added) and individual mortality was assessed every 24 hours. The flies were sexed at death, and the exact cohort size was calculated from the complete recorded deaths.

Mean longevity was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The model used for the data is described as follows: Let y_{ijkm} be the longevity for regime – i (i =1 (AUC), 2 (UX), 3 (RUX)), treatment-j (j=1 (urea), 2(banana)), population – k (k=1,.., 30) and individual – m (k=1,.., n_{jk}). An LME model for longevity is,

$$y_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where δ_s = 0, if s = 1, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively. The

Least-Squares means (lsmeans) package in R was used to calculate p-values from the multiple comparisons. A Tukey's range test was used to correct for multiple comparisons. Males and females were analyzed separately and pooled.

Larva to adult development time and viability in urea and banana

At generation 77 of RUX relaxed selection, the five AUC, UX and RUX populations were assayed to determine development time as well as larva to adult viability in urea and banana food. In this experiment, the time from larvae hatching from egg to adult eclosion from pupae was studied. Prior to the experimental collect, all 15 populations were maintained on banana food for two generations. Eggs from the AUC, UX and RUX populations were collected on non-nutritive agar. From each agar plate, 55 first-instar larvae were transferred to polystyrene vials with either banana or urea food (16g urea/L food). 10 vials per replicate were assayed. Vials were checked every six hours after the first adult flies eclosed, and all eclosed flies were counted and sexed by microscope.

Larva to adult development time was analyzed using a linear mixed-effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The model used for the data is described as follows: Let y_{ijkm} be the development time for regime – i (i =1 (TDO) or 2 (TSO)), treatment-j (j=1 (urea), 2(banana), population – k (k=1,.., 10) and individual – m (m=1,.., n_{jk}). A LME model for time to eclosion is,

$$y_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where δ_s = 0, if s = 1, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively. The Least-Squares means (lsmeans) package in R was used to calculate p-values from the multiple

comparisons. A Tukey's range test was used to correct for multiple comparisons. Males and females were analyzed separately and pooled.

Larva to adult viability was analyzed using a linear mixed effects model (LME) in the R-project for statistical computing (https://www.r-project.org). The model used for the data is described as follows: Let y_{ijkm} be the development time for regime – i (i =1 (TDO) or 2 (TSO)), treatment-j (j=1 (urea), 2(banana), population – k (k=1,.., 10) and vial – m (m=1,.., n_{jk}). A LME model for larvae to adult viability is,

$$y_{ijkm} = \alpha + \delta_i\beta_i + \delta_j\gamma + \delta_i\delta_j\pi + b_k + \varepsilon_{ijkm}$$

where δ_s = 0, if s = 1, and 1 otherwise, and b_k and ε_{ijkm} are assumed to be independent random variables with a normal distribution with zero mean and variances σ_1^2 and σ_2^2 respectively. The Least-Squares means (lsmeans) package in R was used to calculate p-values from the multiple comparisons. A Tukey's range test was used to correct for multiple comparisons. Males and females were analyzed separately and pooled.

RESULTS

TSO and TDO Experiments

TSO and TDO Mortality and Mean Longevity

Morality rates were measured in the TSO and TDO populations. From the Gompertz model fit to our mortality data, A is the age-independent parameter which gives a measure of the baseline mortality rate. α is the age-dependent parameter which gives a measure of the rate of aging. The TDO populations have lower values for the parameters A and α compared to the TSO populations. These differences are significant for A (p=0.0001; Fig. 4.1; Table S4.1; See Figure S4.2 for survivorship plots) but are not significant for α (p=0.945; Figure 4.1; Table S4.1). In addition, the TDO populations show a greater break-day (bd) compared to the TSO populations (p<0.0001; Figure 4.1; Table S4.1).

When analyzing mean longevity, the TDO populations live ~7 days longer than the TSO populations (p=0.0009; Figure 4.2; Table S4.2). These significant differences are observed in both males and females. As seen in Figure 4.2A, the observed difference in mean longevity is comparable to the peak difference in mean longevity observed when the populations were under directional selection (when they were maintained as C's and D's).

TSO and TDO Development Time: Larva to Adult

Larva to adult development time was measured in the TSO and TDO populations. The TDO populations take about one hour longer to eclose from pupa compared to the TSO populations, however this difference is not significant (p=0.66; Fig. 4.3; Table S4.3).

TDO and TSO Adult Age-specific Fecundity

Age-specific fecundity was monitored in the TDO and TSO populations. The TDO populations show a greater number of eggs laid per surviving female (m_x) compared to the TSO populations in the second interval (days 18-20 from egg) ($p=0.021$; Figure 4.4; Table S4.4). This is the interval just prior to these populations' reproductive window (days from egg 20-21). The reproductive window is the period when eggs are used from these populations for the next generation. All other intervals from the analysis do not show statistically significant differentiation ($p>0.05$).

TDO and TSO Fungal-resistance

Mortality after exposure to the fungus *Beauveria bassiana* was monitored in the TDO and TSO populations. No difference was observed in the ability of these populations to survive after exposure to the fungus ($p= 0.123$; Fig. 4.5).

AUC, UX, and RUX Experiments

AUC, UX, RUX Mean Longevity

Mean longevity was analyzed in the AUC, UX and RUX lines to determine how these populations survive after exposure to urea as larvae. For generation 0, there is no significant difference between mean longevity of the AUC and UX populations on banana food, however a significant difference exists on urea food with the UX populations living ~16 days longer (Table 4.1; Table S4.5-4.6). This differentiation remained the same for generations 14 and 31. After 15 generations of relaxed selection, we see no difference between the UX and RUX populations, while the AUC and RUX populations remain strongly differentiated (Table 5.1; Table S4.5-4.6).

After 31 generations, this same pattern exists with no change in the RUX populations' ability to survive after exposure to urea as larvae. (Table 4.1; Table S4.5-4.6)

AUC, UX, RUX Development Time and Viability

Larva to adult development time and viability was monitored in the AUC, UX, and RUX populations after 77 generations of relaxed selection. There is no significant difference in development time between AUC and UX, AUC and RUX, and UX and RUX in banana food ($p>0.05$; Table 4.2). In urea, the RUX and UX populations have ~11-hour difference in development time with the RUX populations taking longer to develop. However, this difference is not quite significant after a Tukey correction. ($p=0.07$; Table 4.2). In addition, The AUC and RUX populations have no difference in development time ($p=1$; Table 4.2). The RUX populations seem to be converged upon the AUC controls for this measure.

There is no difference in viability rates in urea food for the RUX and UX populations after almost 80 generations of relaxed selection ($p=0.83$; Table 5.3). Moreover, the RUX populations have a 23% higher viability in urea food compared to the AUC populations ($p<0.0001$; Table 4.3), while the UX populations have a 25% higher viability compared to the AUC populations ($p<0.0001$; Table 4.3). The UX and RUX populations have a slightly lower viability in banana food compared to the AUC populations. This is not significant for the UX but is significant for the RUX ($p=0.045$; Table 4.3). The RUX populations have not changed in their viability in urea food.

DISCUSSION

Relaxation of Desiccation Selection

We did not observe any differences between the TDO and TSO populations for larva to adult development time and the ability to survive after exposure to a fungus. It is important to note that these phenotypes were not measured during active desiccation and starvation selection of the TDO and TSO populations, however we would have expected these traits to be differentiated during active selection. Phillips et al. (in review) found no signs of differences in the ability of the TDO and TSO populations to survive in a desiccated environment. Desiccation resistance was highly differentiated in these populations prior to relaxed selection (Rose et al. 1992; Gibbs et al. 1997; Djawdan et al. 1998; Archer et al. 2007; Figure 4.2). In addition, Phillips et al. (in review) found no differences in starvation resistance between the TDO and TSO populations, a trait that that was found to be correlated with increased desiccation resistance (Djawdan et al. 1998; Figure 4.2). Significant differences in female fecundity were limited to a single window spanning day 18 to day 20 from egg (day 9-11 from eclosion) with TDO populations producing significantly more eggs. There was no difference in mean fecundity across the 14 days we assayed. Chippindale et al. (1993) found that D populations had significantly higher fecundity compared to the C populations. However, their experiment only measured early fecundity (day 3-5 from eclosion).

Most notably, the TDO populations lived approximately seven days longer than the TSO populations. Previous work has shown that selection for increased longevity is associated with increased desiccation resistance (Service et al. 1985; Graves et al. 1992), and furthermore direct selection for increased desiccation resistance was associated initially with increased longevity (Rose et al. 1992). Further work with sustained selection for desiccation resistance revealed a more complex relationship, with the greatest benefits for longevity accruing at intermediate levels of

increased desiccation resistance (Archer et al. 2003; Phelan et al. 2003). It is surprising and perhaps noteworthy that the longevity difference between the TDO and TSO populations is similar to the longevity difference that they exhibited at their peak of differentiation for this character, particularly for females (Rose et al. 1992; Chippindale et al. 1993). Furthermore, when desiccation selection proceeded to very high levels of desiccation resistance in the D (ancestral to TDO) populations, their differentiation for longevity relative to the C (ancestral to TSO) populations fell from this peak. In the case of the present TDO and TSO populations, the differentiation of desiccation is now gone, at least at the level of statistical detectability. Yet the longevity difference has returned to its former peak level. Despite the seven-day difference in longevity, Phillips et al. (in review) found only limited evidence of genetic differentiation between the TDO and TSO populations. However, recent work has shown that the level of statistical power used in this study may not be large enough to detect residual differentiation between these populations (Graves et al. 2017). Lastly, Philips et al. (in review) found that a lack of genetic variation in these populations does not seem to be the reason for the failure of convergence in longevity after ~230 generations, as they have not apparently suffered any loss of genetic variation.

Relaxation of Urea Selection

After ~80 generations of relaxed selection, we expected to see the RUX populations converge upon the AUC controls. However, we see no signs of convergence in these populations. Adult mean longevity after exposure to urea during larval development did not differ between the RUX and UX populations after 31 generations. This pattern is similar to the lack of initial response seen in populations relaxed from desiccation selection (Passananti et al. 2004). In addition, we see no difference in larva to adult viability in urea food between the RUX and UX populations after

77 generations. Perhaps after more generations of relaxed selection, we will see convergence for adult mean longevity after larvae exposure to urea.

Larva to adult development time in urea food was not statistically different between any of the populations after using a Tukey's range test to correct for multiple comparisons. In particular, the AUC and RUX populations show no difference in development time. Without the Tukey correction, the 11-hour difference in development time between the AUC and UX populations becomes significant ($p<0.05$). This is also the case for the RUX and UX comparison. The RUX populations are taking ~11 hours longer to develop in urea food compared to the UX populations. Taken together, the AUC and RUX populations have converged for development time in urea food.

Joshi et al. (1996b) found that the underlying genetics of urea tolerance is largely dominant. This suggests that the response to relaxed selection should be stronger, but we do not find evidence of this for the phenotypes studied, aside from development time in a urea-supplemented environment. Relaxed selection should be monitored longer to determine the extent to which the RUX populations converge upon the AUC populations. A plausible hypothesis is that urea selection produces genetic changes analogous to those of desiccation selection, with a slower initial response to reverse selection than is observed for starvation selection or demographic selection regimes (Teotonio and Rose, 2000; Burke et al., 2016). If that is the case, much of the differentiation between formerly urea-selected stocks and their controls could be gone in another two or three hundred generations of relaxed selection, as observed for the formerly desiccation-selected stocks.

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FIGURES

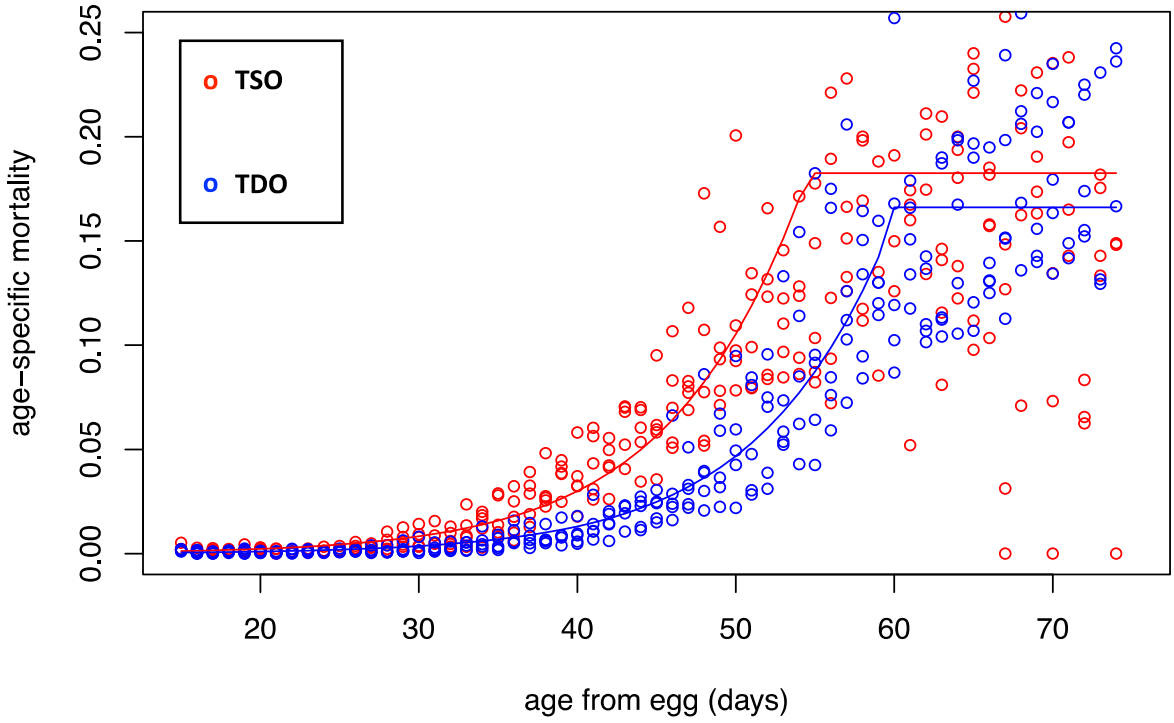


Figure 4.1. Age-specific mortality rates across the five TDOs and TSOs. Replicates shown in blue and red open circles respectively. The data was fitted by a two-stage, three-parameter Gompertz model. Fitted lines for the TDO and TSO populations are shown in blue and red respectively. TDO's are living significantly longer than the TSO's.

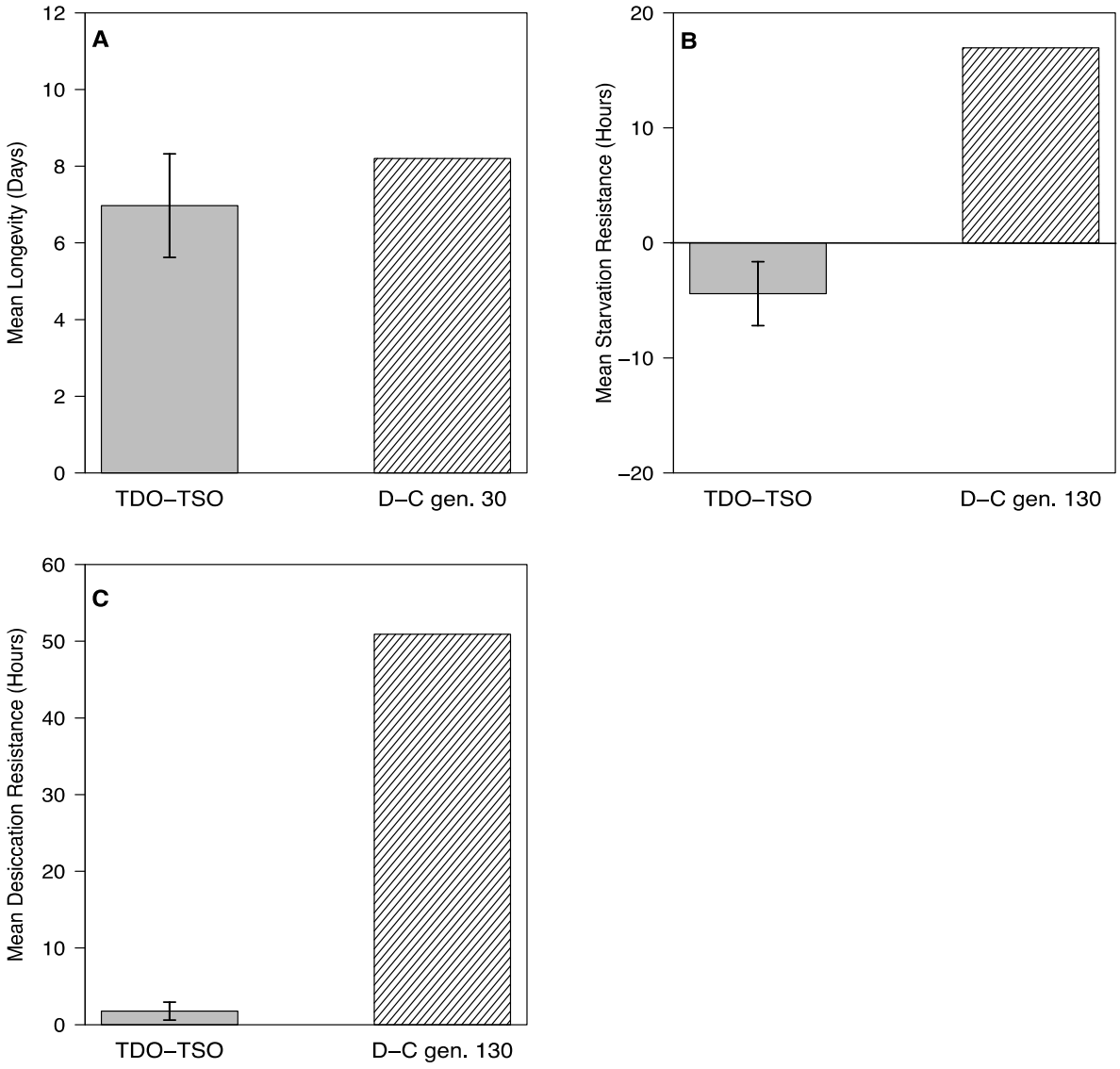


Figure 4.2. Historical and current starvation resistance, desiccation resistance and mean longevity data from females in the desiccation selected and control lines. (**a**) Difference in average longevity between the selected and control populations. Difference in mean longevity was highest early in desiccation selection (near generation 30) (Rose et al. 1992) and then decreases to near zero toward the end of selection (Archer et al. 2003). (**b**) Difference in mean survival time in a starvation environment of flies at age 15 days from egg. The TSO and TDO populations are not significantly different in starvation resistance. However, starvation resistance was significantly different during generation 130 of active desiccation selection (Djawdan et al. 1998; Phelan et al. 2003; Philips et al. in review.). (**c**) Difference in mean survival time in a desiccation environment of flies at age 15 days from egg. Desiccation resistance differences were highest toward the end of selection (generation 200) and have since returned to close to zero (Djawdan et al. 1998; Archer et al. 2003; Philips et al. in review). Error bars are mean ± 1 SEMD.

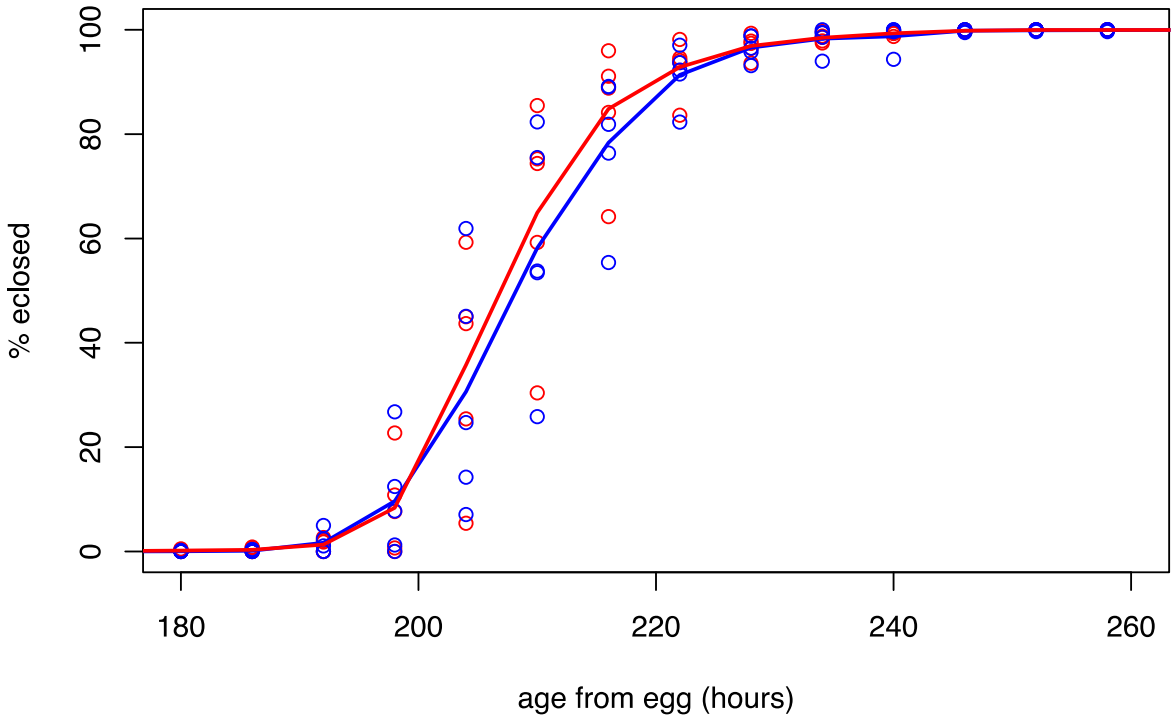


Figure 4.3. Time to eclosion in the TDO and TSO populations. Points represent the percentage of the total cohort of flies eclosed each collection interval for each replicate. Lines represent averages across replicates. TSO populations are represented by red lines and open red circles, while the TDO populations are represented by blue lines and open blue circles. The TDO's are statistically converged upon the TSO's for this development time measure.

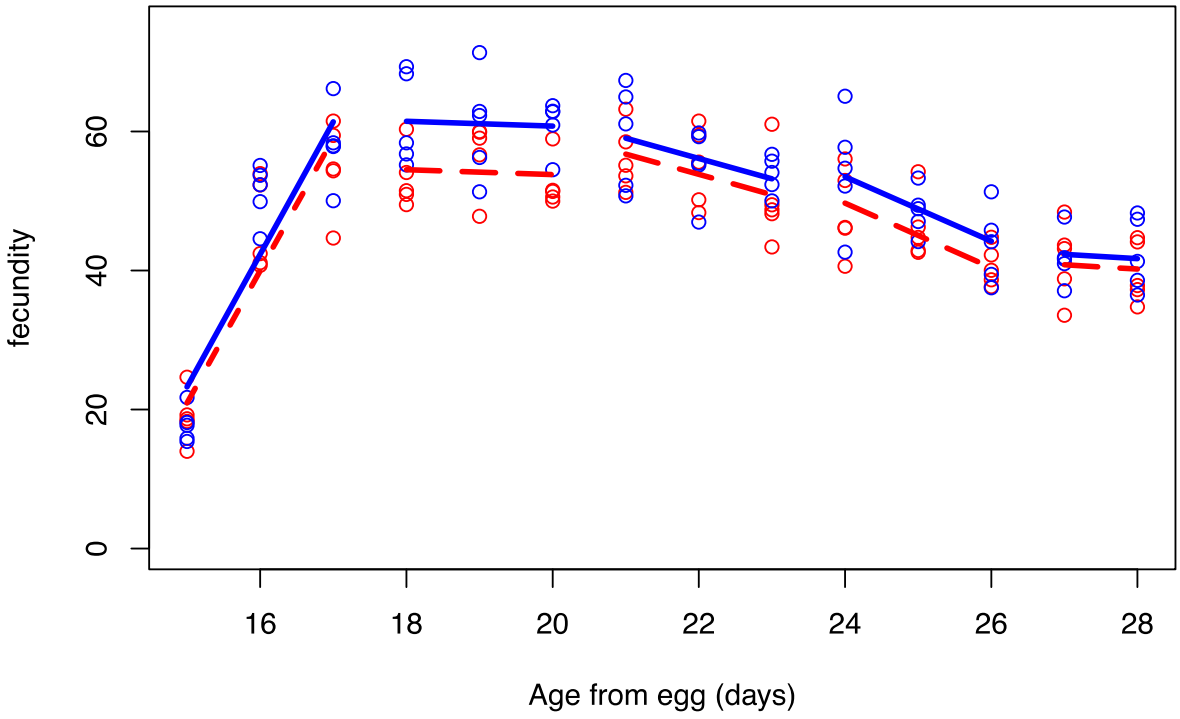


Fig 4.4. Adult age-specific fecundity from the TDO and TSO populations. Open red circles and dashed red lines represent average eggs laid per female per day as a function of age in the five TSO populations. Open blue circles and solid blue lines represent the five TDO populations. TDO populations have significantly higher fecundity in the second interval (p=0.021). All other intervals are not significant.

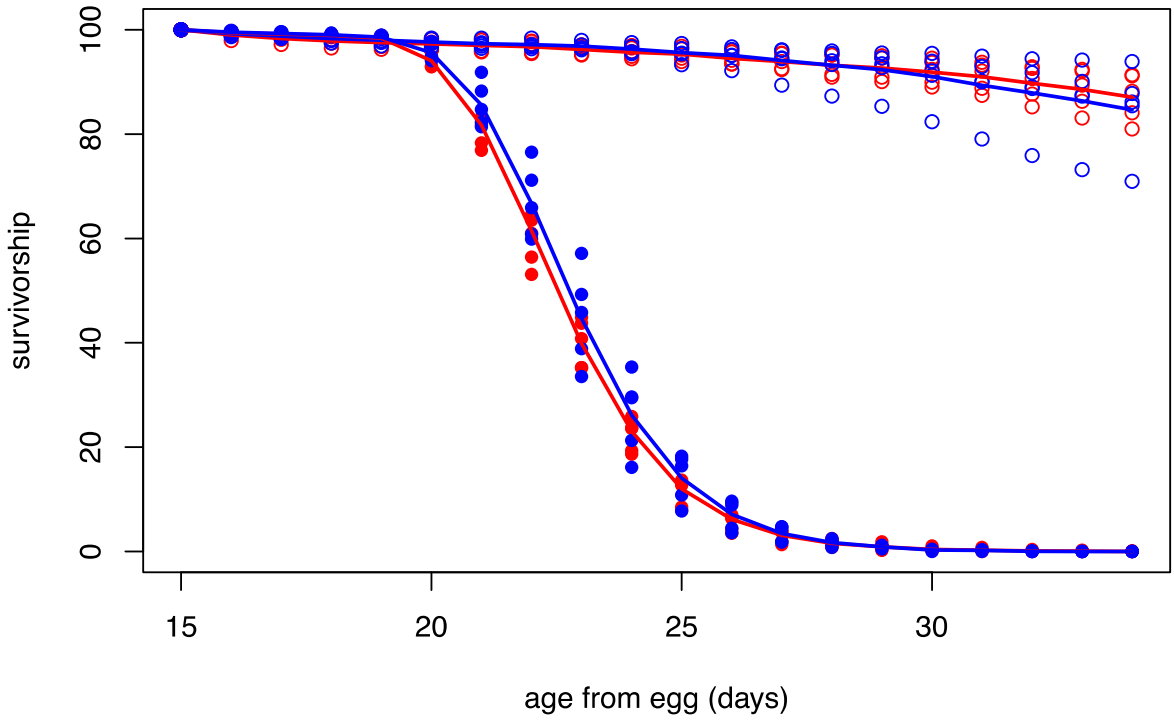


Figure 4.5. Adult survivorship (l_x) from the TDO and TSO fungal resistance experiment. The control populations are represented by open points and the populations exposed to fungus are represented by closed points. TSO and TDO points are shown in red and blue respectively. Trend lines show averages across all replicates and across three experiments. Fungal resistance is not significantly different between the TDO and TSO populations. (p=0.123)

TABLES

Table 4.1. Mean longevity after 0, 14 and 31 generations of relaxed selection in the RUX populations. Values are difference in mean longevity (days) for sexes pooled. No response is seen after 31 generations of relaxed urea selection. *p<0.01 **p<0.001 ***p<0.0001

	Generation 0		Generation 14		Generation 31	
Regime	Banana	Urea	Banana	Urea	Banana	Urea
AUC-UX	-1.53	-16.15***	1.04	-14.83***	-0.007	-16.66***
AUC-RUX	NA	NA	-4.26	-16.29***	1.44	-18.15***
UX - RUX	NA	NA	-5.29	-1.45	1.45	0.78

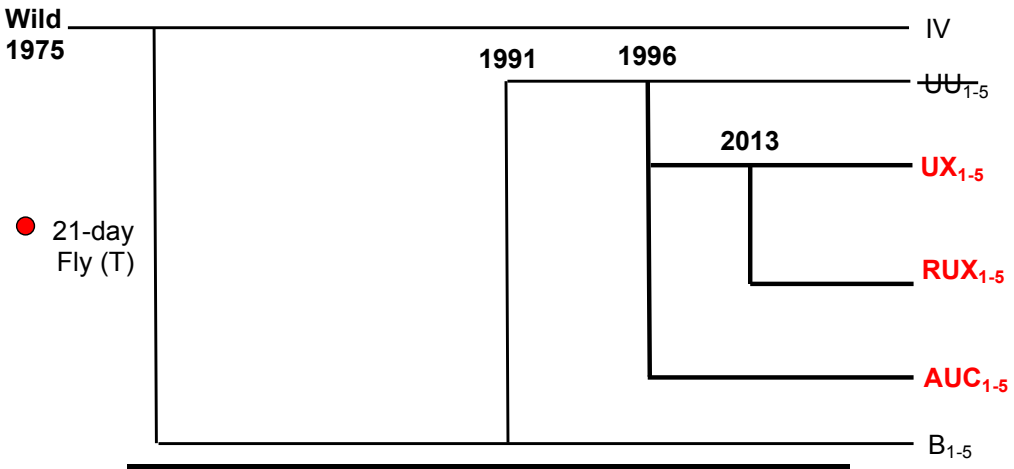
Table 4.2 Development time after ~80 generations of relaxed selection in the RUX populations. Values are difference in mean development time (hours). No significant differences are observed.

	Sexes Pooled		Females		Males	
Regime	Banana	Urea	Banana	Urea	Banana	Urea
AUC-UX	-6.79	10.89	-5.97	-0.18	-7.67	11.91
AUC-RUX	-9.61	-0.015	-9.16	0.33	-9.95	-0.11
UX-RUX	-2.81	10.90	-3.18	-9.84	-2.29	-12.02

Table 4.3 Percent viability after ~80 generations of relaxed selection in the RUX populations. Values are difference in mean development time (hours). No response is seen after 77 generations of relaxed selection. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

Regime	Banana	Urea
AUC – UX	6.80%	-24.69% ****
AUC - RUX	8.15% *	-22.84% ****
UX - RUX	1.35%	1.85%

SUPPLEMENTAL MATERIALS



	Egg → Day 14	Day 14 → Day 21
AUC_{1-5}	Banana	Banana
UX_{1-5}	Urea	Banana
RUX_{1-5}	Banana	Banana

Figure S4.1 Derivation of the urea-tolerant (UX), unselected controls (AUC), and reverse-selected populations (RUX). These three selection regimes are five-fold replicated and maintained at large population sizes (~1000). The UX and AUC populations were derived from the five UU populations (now extinct) (Joshi and Mueller 1996a). The UU populations were derived from the B populations (Rose 1984). The RUX populations were derived from the five UX populations. All populations except for the B and IV populations are maintained on a three-week life-cycle.

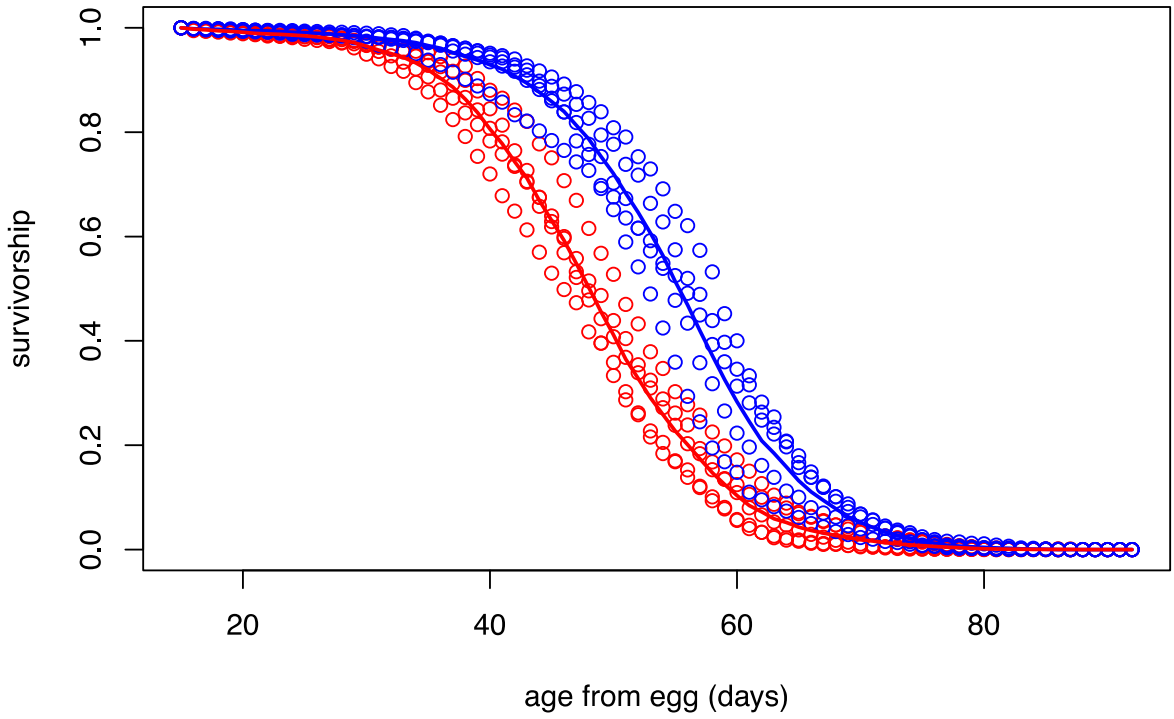


Figure S4.2. Survivorship (l_x) for the TDO and TSO populations. TSO populations are represented by red lines and dots. TDO populations are represented by blue lines and dots. Lines represent pooled survivorship. TDO populations live significantly longer than TSO populations.

Table S4.1. TSO vs. TDO adult age-specific mortality using the two-stage, three parameter Gompertz model. Significant p-values are bolded.

Parameter	Difference	Std. error	t-value	D/F	*p*-value
A	0.00009	0.000022	4.0	1777	**0.0001**
α	0.00032	0.0047	0.07	1777	0.945
bd	-5.73	0.927	-6.18	1777	**<0.0001**

Table S4.2. TSO vs. TDO mean longevity analysis. Significant p-values are bolded.

Difference (days)	Std. error	t-value	D/F	*p*-value
-6.97	1.35	-5.147	8	**0.0009**

Table S4.3. TSO vs. TDO mean development time. Significant p-values are bolded.

Difference (hours)	Std. error	t-value	D/F	*p*-value
-1.16	2.62	0.45	8	0.66

Table S4.4. TSO vs. TDO age-specific fecundity. Significant p-values are bolded.

Age range	Difference (intercept)	Std. error	t-value	D/F	*p*-value
15-17	-2.33	2.43	-0.958	8	0.3663
18-20	-6.99	2.43	-2.878	8	**0.0206**
21-23	-2.29	2.43	-0.944	8	0.3730
24-26	-3.82	2.43	-1.571	8	0.1549
27-28	-1.50	2.79	-0.537	8	0.6058

Table S4.5. Mean longevity after 0, 14 and 31 generations of relaxed selection in the RUX populations. Values are difference in mean longevity (days) for females. No response is seen after 31 generations of relaxed urea selection. *p<0.01 **p<0.001 ***p<0.0001

	Generation 0		**Generation 14**		**Generation 31**	
Regime	**Banana**	**Urea**	**Banana**	**Urea**	**Banana**	**Urea**
AUC-UX	-2.96*	-16.21***	1.08	-14.83***	-0.007	-16.66***
AUC-RUX	NA	NA	-4.39	-16.29***	1.44	-18.15***
UX - RUX	NA	NA	-5.47	-1.45	1.45	0.78

Table S4.6. Mean longevity after 0, 14 and 31 generations of relaxed selection in the RUX populations. Values are difference in mean longevity (days) for males. No response is seen after 31 generations of relaxed urea selection. *p<0.01 **p<0.001 ***p<0.0001

	Generation 0		**Generation 14**		**Generation 31**	
Regime	**Banana**	**Urea**	**Banana**	**Urea**	**Banana**	**Urea**
AUC-UX	0.01	-16.05***	0.98	-14.83***	-0.007	-16.66***
AUC-RUX	NA	NA	-4.21	-16.29***	1.44	-18.15***
UX-RUX	NA	NA	-5.19	-1.45	1.45	0.78

CHAPTER 5

Summary and Conclusions

Research in the field of experimental evolution has revealed that it takes surprisingly few generations for populations to adapt to new conditions, at least at ages when natural selection is intense (Matos et al. 2000; Simões et al. 2007; Simões et al. 2009; Fragata et al. 2014). This is contrary to the thinking of those like Charles Darwin, who famously emphasized the extreme gradualness of the action of natural selection in producing evolutionary change (Darwin 1859). The speed and effectiveness of experimental evolution has convinced some that humans are likely to be well adapted to agricultural diets and activity levels, at least among populations of Eurasian ancestry. Evolutionary biologist Marlene Zuk has been a proponent of this idea, arguing that the last 200-400 generations of large-scale agriculture has been enough time to adapt humans with agricultural ancestry to an organic agricultural diet (Zuk 2013).

Current diets and activity patterns in the industrialized West of course do not match the Eurasian history of selection for adaptation to agricultural conditions. But it is reasonable to suppose that a reversion to "organic" or "natural" agricultural foods and activity patterns should significantly improve human health. However, even this conclusion is not correct for populations that have only recently adopted agricultural diets, as illustrated by work with the aboriginal populations of Australasia (Lindeburg 2010; Rowley et al. 1997). Populations that have recently adopted the agricultural diet have been shown to exhibit dramatic declines in health (Larsen 1995). "Paleo" enthusiasts, who apparently know little about recent research on evolution, have argued that adopting a Paleolithic hunter-gather diet would alleviate many chronic disorders, not only in newly agricultural groups (Jönsson et al. 2009), but even in individuals with long agricultural ancestry. They believe that humans can best optimize metabolism and physiology when they consume a diet more like that of our hunter-gatherer ancestors before the advent of agriculture.

The verbal argument just sketched ignores the age-specificity of adaptation in populations with age-structure and age-specific genetic effects. In general, as was introduced in chapter 1 and expanded on in subsequent chapters, adaptation is age-specific, with Hamilton's forces of natural selection leading to much greater adaptation at earlier ages than later ages. This is how evolutionary biologists explain the existence of aging in the first place (Hamilton 1966). When the pattern of Hamilton's forces is changed, aging can be decelerated or accelerated simply by shifting the windows of reproduction in the life-cycle imposed on laboratory cultured populations that are not inbred (Rose 1984).

At later adult ages, when the forces of natural selection are weak, natural selection will only slowly produce adaptation to a selective environment that is not evolutionarily ancient. In effect, late adult ages will feature relics of adaptation to long past environments. Chapter 2 of this dissertation tested this hypothesis in two dietary transition experiments with outbred lab populations of *Drosophila melanogaster*. The first experiment featured one longstanding diet, banana without supplemented urea, being replaced by another long-standing diet, urea supplemented banana food. Results showed that the gain of adaptation to the urea food and the loss of adaptation to the banana food was strongest at early ages, as expected under the model of age-specificity.

The second experiment featured populations that used to consume a diet incorporating rotting apples but have since spent many generations on a diet based on banana with live yeast. These populations had been exposed to the apple diet in the wild for roughly 10,000 generations. But soon after they were brought into the lab, they were given a banana diet for ~1000 generations. In addition, these flies were assayed on a novel diet that featured oranges. The results showed that younger adult flies fared well on the banana diet, while older adult flies fared better on the apple

diet. Our interpretation is that this pattern arose because the forces of natural selection acting on these populations had weakened enough to forestall sufficient adaptation to the banana food provided more recently in their evolutionary history. Flies performed better on the banana diet at earlier ages compared to the novel orange diet, but performance on orange and banana diets was similar at later ages. This again suggested a lack of adaptation to banana food at later adult ages in these populations.

If adaptation to the banana diet is dependent on when the forces of natural selection decline, it would be expected that populations evolving under a culture regime that allowed reproduction only at later ages should have adaptation to the banana food further into adulthood. This hypothesis was tested using populations with the same dietary transition from apple to banana, but that have been also had a reproductive window at later ages for hundreds of generations. As predicted, these populations performed significantly better on the banana diet compared to the apple diet at ages prior to their much later fall in the forces of natural selection. No significant differences were observed at later ages between cohorts given banana or apple diets. Though these populations performed somewhat better on the apple diet at the last ages of comparison, that improved performance was not statistically significant.

Experiments switching among diets at various adult ages could give a clearer picture as to optimal diet as a function of adult age. Santos et al. (in prep) performed banana-orange diet switch experiments using the same assay procedures as were performed in this work. They found that switching diets during adult-life had statistically detectable effects in *D. melanogaster* cohorts. A future experiment could assay populations on banana food at early ages and apple food at later ages. This treatment should lead to greater total reproductive output over all ages, compared to cohorts exposed to either banana food or apple food from larvae to adult death. In addition, cohorts

could be given apple food at earlier ages and banana food at later ages. This treatment should perform worst. The results from chapter 2 were obtained from diet experiments that monitored populations with inadvertent diet transition histories. That is, we did not monitor forward selection on the banana diet or on the urea diet directly. Future experiments could directly monitor age-dependent adaptation in populations during forward and reverse selection involving diets.

Chapter 3 combined the approach of chapter 2 with a pharmaceutical approach to healthspan extension. Two botanicals, derived from *Rhodiola rosea* and *Rosa damascena,* have been extensively studied for their anti-aging properties (Jafari et al. 2007; Jafari et al. 2008). Both botanical extracts have extended *D. melanogaster* mean lifespan without impairing other physiological functions, including fertility (Jafari et al. 2007; Jafari et al. 2008). This chapter evaluated the effects of combining both diet and supplement manipulations on *Drosophila* healthspan. The results revealed that when the banana diet is supplemented with *Rosa damascena,* fly populations live longer. However, when supplementing the apple diet with *Rosa damascena,* an antagonistic effect on survivorship was observed. On the other hand, *Rhodiola rosea* added to banana food extended lifespan by 17% and mimicked the effects of the apple diet, with lower performance at early ages and higher performance at later ages compared to the control banana treatment.

There is a widespread belief that supplementing diets with a plethora of vitamins and other nutraceuticals will enhance healthspan. However, many studies in humans and mice have not found combined supplementation to be useful for preventing diseases and increasing longevity (Macpherson et al. 2013; Watkins et al. 2000; Park et al. 2011; Spindler 2012; Spindler 2014). In fact, Spindler et al. (2014) found that some complex nutraceuticals decrease lifespan in mice. More recently, Jenkins et al. (2018) performed a meta-analysis in humans and found that antioxidant

mixtures and niacin resulted in an increase in all cause-mortality. This sparked the "Poisoned Chalice" hypothesis outlined in Chapter 1 of this dissertation (Rutledge and Rose 2015). Perhaps novel combinations of supplements can elicit adverse physiological responses. Chapter 3 tested this hypothesis by exposing fruit flies to *Rosa damascena* and *Rhodiola rosea* in combination. When these botanicals were combined an antagonistic effect was observed. The Poisoned Chalice hypothesis should be tested further using other combinations of compounds assayed in different diet backgrounds.

Chapter 4 of this dissertation studied the effects of evolutionary history on phenotypic convergence in populations selected for extreme desiccation resistance. Experiments with *Drosophila* have shown that laboratory evolution can be rapid and highly repeatable (Matos et al. 2000; Simões et al. 2008; Archer et al. 2003; Burke et al. 2016). These studies suggest that phenotypes are primarily shaped by the most recent selection regimes imposed on a population, and that evolutionary history has little impact. However, reverse evolution studies suggest that reversal back to the ancestral state depends on evolutionary history and the character studied (Teotónio and Rose 2000; Passananti et al. 2004). This chapter used D. *melanogaster* populations that were selected for intense desiccation resistance for ~250 generations and have since been relaxed for an additional ~250 generations. The populations previously selected for desiccation resistance lived seven days longer than their controls, a similar difference to that exhibited at their peak differentiation for mean longevity. Development time, fungal resistance, desiccation resistance, and starvation resistance were not significantly different between these populations. These findings suggest that extreme selection can have long-lasting impacts on phenotypic differentiation for longevity, depending on the character studied.

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