Gut Bacterial Load Associates with Dramatic Declines in Anoxia Tolerance in Young *Drosophila melanogaster* Adults

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved October 2019 by the
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ARIZONA STATE UNIVERSITY

May 2020

ABSTRACT

Anoxia tolerance is strongly correlated with tolerance to heat, desiccation, hyperosmotic shock, freezing, and other general stressors, suggesting that anoxia tolerance is broadly related to stress tolerance. Age affects the capacity of many animals to survive anoxia, but the basis to this ontogenic variation is poorly understood. We exposed adult *Drosophila*, 1, 3, 5, 7, 9, and 12 days past eclosion, to six hours of anoxia and assessed survival 24-hours post-treatment. Survival of anoxia declined strongly with age (from 80% survival for one-day-old flies to 10% survival for 12 day-old-flies), a surprising result since adult fly senescence in *Drosophila* is usually observed much later. In anoxia, adenosine triphosphate (ATP) levels declined rapidly (< 30 min) to near-zero levels in both 1 and 12-day old adults; thus the higher anoxia-tolerance of young adults is not due to a better capacity to keep ATP elevated. Relatively few physiological parameters are reported to change over this age range in *D. melanogaster*, but gut bacterial content increases strongly. As a partial test for a causal link between bacterial load and anoxia tolerance, we replaced food daily, every third day, or every sixth day, and assayed survival of six hours of anoxia and bacterial load at 12 days of age. Anoxia tolerance for 12-day old flies was improved by more food changes and was strongly and negatively affected by bacterial load. These data suggest that increasing bacterial load may play an important role in the age-related decline of anoxia tolerance in *Drosophila*.

ACKNOWLEDGMENTS

I would like to thank numerous members of the Harrison lab for their useful feedback on experimental design as well as Dr. Douglas Lake and Dr. Shelly Haydel for taking the time to serve on my graduate committee and providing their unique interdisciplinary perspectives. This work was supported by an NSF IOS 1256745 to JFH and the School of Life Sciences Undergraduate Research (SOLUR) Program at Arizona State University, Tempe Campus.

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Introduction:

Oxygen is essential for long-term survival of most animal life, yet likely all animals have some capacity to cope with anoxia (Somero et al., 2017; Zhou and Haddad, 2013). While oxygen typically tends to be in adequate supply in the terrestrial environment, in aquatic and semi-solid environments (such as soil or for invertebrates in grain or living within other animals) hypoxia and even anoxia are common (Hoback and Stanley, 2001; Hochachka et al., 1996; Schmitz and Harrison, 2004). Animals vary tremendously in their capacities to tolerate hypoxia/anoxia. A typical mammalian response to anoxia results in rapid organismal paralysis, cellular damage, and death within a matter of minutes (Semenza, 2014). However, some vertebrates such as freshwater turtles can survive months without oxygen (Galli and Richards, 2014). In addition to the variation of anoxia tolerance among species, considerable variation in hypoxia/anoxia tolerance can exist within species due to genetic, environmental and age-related factors (Azad et al., 2011; Callier et al., 2015a; Campbell et al., 2019a; Harrison and Haddad, 2011a; Mariani et al., 2000; Parer, 1998; Resnik-Docampo et al., 2018; Zhou et al., 2007; Zhou et al., 2008). In this study, I test for possible mechanisms responsible for age-related variation in anoxia-tolerance in adult *Drosophila melanogaster*.

Despite the fact that anoxia plays an important role in heart disease, stroke, and peripheral artery disease (PAD), both the mechanisms causing cell death and the physiological processes responsible for within-species variation in anoxia tolerance remain poorly understood (Armstrong et al., 2011; Campbell et al., 2018; Campbell et al.,

2019b; Ma et al., 2001; Mariani et al., 2000; Opie, 1991; Ravn et al., 2019; Sendoel and Hengartner, 2014). A typical response to anoxia begins with a rapid exhaustion of ATP caused by blockage of mitochondrial ATP production due to lack of oxygen (Cherubini et al., 2005; Murphy and Steenbergen, 2008). Organismal paralysis typically occurs within seconds of initial anoxia exposure (Semenza, 2014). Cellular pH drops as an organism's metabolism shifts toward anerobic glycolysis to compensate for a lack of ATP (Murphy and Steenbergen, 2008). An excessive drop in pH can interfere with protein folding (Feala et al., 2007; Murphy and Steenbergen, 2008). Lack of ATP inhibits the cellular Na^+/K^+ and Ca^{2+} ATPases, causing membrane depolarization due to K^+ leakage. Depolarization eventually leads to an influx of extracellular Ca^{2+} through voltage-sensitive Ca^2+ channels (Cherubini et al., 2005; Murphy and Steenbergen, 2008), which can activate Ca^{2+}-stimulated enzymes including proteases, lipases, nucleases, and protein kinases, leading to cellular damage, reactive oxygen species production (ROS) and proinflammatory gene expression (Cherubini et al., 2005). Damage can also occur following the reintroduction of oxygen (reperfusion injury), likely due to ROS generation, causing further stimulation of inflammation and apoptotic and necrotic cascades (Cherubini et al., 2005; Murphy and Steenbergen, 2008).

Anoxia-tolerant vertebrate species such as *Chrysemys picta* (painted turtles) and *Carassius carassius* (crucian carp) have a wide array of adaptations that generally allow them to maintain ATP levels during long periods of anoxia (Galli and Richards, 2014; Nilsson and Lutz, 2004). These include the capacity to strongly-suppress metabolic rate, high capacities for anaerobic metabolism, and elevated capacities to cope with the

cellular stresses, such as higher antioxidant and chaperone concentrations (Galli and Richards, 2014). Insects tend to be extremely anoxia tolerant relative to mammals, generally being able to survive many hours of anoxia (Harrison, 2015; Harrison and Haddad, 2011b; Hoback and Stanley, 2001; Schmitz and Harrison, 2004). One possible evolutionary explanation for the generally high-anoxia-tolerance of insects is that, due to their small body size, insects are at a much higher risk of drowning (Benasayag-Meszaros et al., 2015). More mechanistically, the generally high capacities of insects to survive anoxia may also be associated with the fact that the tracheal respiratory system allows oxygen renewal by diffusion when the anoxic period ends. The generally high anoxia-tolerance of insects has also been linked with suppression of metabolism due to paralysis, strong capacities for anaerobic metabolism, upregulation of heat shock proteins, and accumulation of protective metabolites (Armstrong et al., 2009; Benasayag-Meszaros et al., 2015; Campbell et al., 2018; Campbell et al., 2019a; Campbell et al., 2019b; Dawson-Scully et al., 2010; Ravn et al., 2019; Zhao and Haddad, 2011). However, unlike classic anoxia-tolerant vertebrates, most insects do not maintain ATP levels during anoxia (Hoback and Stanley, 2001; Wegener, 1993; Campbell et al. 2018).

While progress is being made in understanding the mechanisms explaining large-scale variation across species or clades in anoxia tolerance, much less is known about the causes of variation in anoxia tolerance among individuals of the same species. Age has been well-documented to affect intraspecies anoxia tolerance (Mariani et al., 2000; Podrabsky et al., 2007; Robertson et al., 2009). In humans and other vertebrates, there is a strong decline in the capacity of tissues to tolerate anoxia with age, and infants are

much more likely to survive an anoxic event than adults (Parer, 1998). Conversely, in *Drosophila melanogaster*, adults survive eight times longer in anoxia than larvae (Callier et al., 2015a). Senescence is also likely to play a role in anoxia-tolerance, as stress-tolerance generally tends to decline with adult age in animals (Benasayag-Meszaros et al., 2015; Rera et al., 2012). *Drosophila melanogaster* adults have been documented to exhibit senescence as evidenced by decreased phototaxis score, longer time of recovery from chill coma, and greater mortality in response to starvation/desiccation stress; however, in general, decreased function is observed at ages of three weeks or more (Carbone et al., 2016; Carnes et al., 2015; Semenchenko et al., 2004). However, some physiological parameters such a egg-laying rate peak in early adulthood (4-7 days of age), and decline significantly even by only two weeks of adulthood (Miller et al., 2014). As part of our prior study comparing the anoxia-tolerance of adult and larvael *D. melanogaster* (Callier et al., 2015b; Campbell et al., 2018), I compared the anoxia tolerance of 1 and 12 day-old flies, expecting to find minimal differences. Instead, I unexpectedly found strongly reduced anoxia-tolerance in 12-day old flies (see below). This finding caused us to carefully explore the effect of age on anoxia-tolerance over this age range, and to test two possible explanations for this decline in anoxia tolerance with age: a decrease in the capacity to conserve ATP levels, and accumulation of bacteria in the gut.

The gut microbiome plays multiple physiological roles including affecting responses to stress (Benasayag-Meszaros et al., 2015; Cho and Blaser, 2012). The microbiome has been shown to affect thermal tolerance and other general stress

tolerances that tend to be correlated with anoxia tolerance (Clark et al., 2015; Moghadam et al., 2018). Additionally, imbalances in the gut microbiome have been linked to many diverse diseases including asthma, obesity, and heart disease (Huttenhower et al., 2012). Regarding the microbiome and aging, mammals and *Drosophila* are similar (Broderick and Lemaitre, 2012; Broderick et al., 2014; Feala et al., 2007; Feala et al., 2009), with the total quantity of bacteria in the gut (bacterial load) increasing while intestinal function declines during senescence (Ferguson et al., 2018; Rera et al., 2012; Resnik-Docampo et al., 2018; Yatsunenko et al., 2012). Intestinal dysfunction has been linked to increased tissue damage and inflammation following intestinal ischemia in mice (Kinross et al., 2009). Pretreatment with erythromycin, a common macrolide antibiotic, increases stroke tolerance up to three-fold in rats (Brambrink et al., 2006), suggesting that decreasing bacterial load may enhance anoxia tolerance. In *D.* melanogaster, and likely other holometabolous insects, bacterial load increases dramatically over the first week of adult development (Blum et al., 2013). As this is one of the few parameters which we could find in the literature that change strongly over the first week of adult age in *D. melanogaster*, and because of the possible association with stress resistance, I manipulated bacterial load and assessed the effect on anoxia tolerance.

Methods:

Study Organism and Rearing Conditions

All the *Drosophila* used in this study were members of the laboratory wild-type Samarkand (SAM) strain (Thurmond et al. 2019). Flies were reared in 250 mL plastic bottles filled with 50 mL of fly food, which was a pre-mixed combination of corn starch,

glucose, and agar (Lab-Express.com, fly food B mix). Fifty milliliters of a 20% tegosept (methyl-p-hydroxy benzoate) solution, a common Drosophila anti-fungal agent, was also added to each liter of food made. To partially control population density, 10 males and females were put into freshly prepared food bottles to create new populations each generation. All flies were reared at 25°C in an insect incubator set to 12-hour light/dark cycles.

Drosophila Collection and Aging

To collect newly eclosed adults for aging experiments, around thirty bottles of *Drosophila* were initiated as described above, ten days prior to the first day of adult collection. On the morning of the ninth day after initiating the population, all adults in the bottles were discarded. The following five mornings, between 9-12 AM, newly-eclosed adults were collected and put into new food bottles, thus ensuring the adults in these bottles were all within 24 hours of the same age. Unless specified otherwise, flies were shifted to bottles with new fly food every five days until adults reached desired age. One day prior to reaching a desired age for an anoxia experiment, adults were CO_2-anesthetized, separated by sex, divided into groups of 20 and placed into 45 mL vials containing 10 mL of fly food.

How does adult age and sex affect survival of six hours of anoxia?

Vials of approximately 20 male or female adults, ages 1, 3, 5, 7, 9, or 12 days after eclosion, were placed into the anoxia treatment chamber, and the number of live flies counted in each vial. The anoxia treatment chamber was a 2 L plastic container

through which humidified nitrogen (80% relative humidity as measured with a Hoboware data logger) was continuously pumped at 3 L min^{-1} though a mass flowmeter. Flies were paralyzed within a minute of the initiation of nitrogen flow, confirming that vials were well-perfused. Vials were placed on their sides to prevent the paralyzed flies from getting stuck in the media. After six hours of anoxia, the lid of the treatment chamber was removed to rapidly restore oxygen to normal, and the vials were undisturbed for 24 hours before counting the surviving flies. Vial were repeatedly tapped and non-moving flies were presumed dead.

Because the flies used at different ages in this experiment came from different vials within our colony, and thus had different parents, I conducted an additional experiment to ensure that the observed effect of age on anoxia tolerance was not due to a chance-difference in the quality of the parents. I collected 300 newly-eclosed flies, and mixed these into a single population. Half of these were immediately CO_2-anesthetized, sorted by sex, and exposed to six hours of anoxia the following day (at one day of age). The remaining flies matured in vials as described above and their survival after six hours of anoxia was tested at 12 days of age.

How does anoxia duration affect the survival of one and twelve-day-old flies?

Groups of newly-eclosed flies were collected and placed into separate bottles. Half of these were sorted by sex, placed into vials of 20, and tested the next day (at one day of age) for survival anoxia durations of 0-12 hours. The other half of the flies were reared as described above to 11 days in age, when flies were CO_2-anesthetized, separated

by sex, and grouped into vials of 20. The next day (at 12 days of age), flies were flies were exposed to anoxia treatment durations ranging from 0 to 12 hours.

ATP Measurement

Vials of one or 12 day-old, same-sex flies were created as described above. Immediately before the anoxia treatment, the flies were transferred without anesthesia to empty 60 mL vials covered with plastic caps. Each plastic cap had around twenty 0.5mm holes to prevent escape yet allow air to flow freely. The vials were perfused with nitrogen at a constant flow rate of 3 L min^{-1} for zero minutes to 12 hours. After the anoxia exposure, the vials containing the flies were placed into liquid nitrogen which penetrated through the holes in the vial lids, allowing fast-freezing. Frozen flies were stored at -80 °C until analyses. A bioluminescence ATP assay was utilized to determine ATP concentration in the pooled group of five adult flies (Campbell et al., 2018). To standardize the ATP values, protein concentration in the homogenate of the five flies was measured with a Bradford protein assay (Kruger, 2002).

How does the quantity of bacteria in a fly affect survival of anoxia?

Flies obtain most of their gut bacteria from ingested food, and changing the food more frequently strongly reduces the number of bacteria in the Drosophila gut (Blum et al. 2013). Therefore, we manipulated food change frequency to alter bacterial load, reasoning that if the accumulation of gut bacteria over the first 12 days of adult life was reducing anoxia tolerance that reduction of bacterial load by frequent food changes should improve anoxia tolerance. Groups of newly-eclosed adults were collected daily

over a four-day period. Each group of newly-eclosed adults was divided into three separate 200 ml bottles containing food. Flies from each of these bottles were moved to a fresh bottle of food either daily, every third day, or every sixth day. When adults reached 11 days of age, they were CO_2 anesthetized, separated by sex, and placed in groups of 20 into 45 ml vials. The next day, five flies from each bottle-transfer interval were assayed for gut bacterial species and load. The remaining 12-day old flies were exposed to six hours of anoxia, allowed to recover for one day, and assessed for survival.

Bacterial Quantity

Groups of five flies from each food change treatment were surface sterilized in 100% ethanol. After drying, fly groups were rinsed two times with sterile phosphate-buffered saline (PBS) and were transferred to sterile 2 mL Eppendorf tubes containing 1250 uL of PBS. Flies were homogenized in the tubes with sterile micro pestles. Ten-fold dilutions were prepared up to $1/10{,}000^{th}$ the original homogenate concentration. The fly homogenate and each dilution was plated on both *Lactobacillus* agar (MRS) and mannitol *Acetobacter* agar (MAN). MAN plates were incubated in atmospheric air at 30°C for two days. MRS plates were incubated in microaerophilic conditions for two days. Microaerophilic conditions were created by sealing plates along with a lit candle in an air-tight glass jar (Guilhot et al., 2018).

Following incubation, plates that contained between 30 and 300 bacterial colonies with the same morphology were counted and used to calculate the average gut bacterial load per fly that corresponded to that specific bacterial morphology. Each bacterial colony type was homogenized in sterile PBS, and serial dilutions of the homogenate were

created using sterile PBS. The bacterial load (CFU, colony forming units per fly) was calculated for the most dilute homogenate that yielded multiple countable colonies:

(**1**):

$$\frac{CFU}{fly} = C \times \frac{DF * VH}{(VP * \#flies)}$$

with C indicating the number of colonies on the plate, DF indicating the dilution factor for that plate, VH indicating the volume of the original homogenate (1250 ml), VP indicating the *volume plated*, or how much of the diluted solution was spread on each plate (100uL), and #flies indicating how many flies were homogenized (5). Although I measured the quantity of bacteria in whole flies, I refer to this as bacterial content of the gut, as prior studies have found that the vast majority of bacteria in *D. melanogaster* are found in the gut (Broderick et al., 2014). All supplies noted to be sterile were sterilized using an autoclave. I continuously verified the effectiveness of the autoclave by culturing samples of blank solutions and swabs from material surfaces prior to use.

Identification of Bacteria

Morphologically distinct colonies from the homogenate-plated MRS and MAN plates were quadrant-streaked on new plates and incubated at the same conditions for two days. After incubation, a single colony of each morphology was taken from the quadrant-streaked plates and transferred to MRS and MAN slants and grown in pure culture. Samples of the pure cultures from each colony morphology were gram-stained to determine cell wall morphology and checked for contamination.

To extract bacterial DNA, a modification of the Kulski et al. method was used (Kulski and Pryce, 1996). An inoculating loop was used to scrape approximately one colony of bacteria off of its corresponding pure culture. Each bacterial mass was suspended in 0.5 mL of sterile 0.5M Tris-HCl (pH 8). The suspensions were centrifuged at 13,000 x g for 5 minutes and decanted. Bacterial pellets were then re-suspended in 0.5 mL 0.5M Tris-HCl (pH 8), centrifuged at the same speed, and decanted again. The bacterial pellets were then re-suspended in 0.1 mL of Tris-EDTA (10 mM Tris–HCl pH 8.0 containing 1 mM EDTA) and boiled in a heat block at 100°C for 10 minutes. To further lyse the cell walls, the samples were frozen and thawed two times using a -80°C freezer. Following the second thawing, the samples were centrifuged at 13,000 x g for 15 minutes. Five microliters of each crude DNA-containing supernatant were transferred to a standard PCR mix containing 1492R and 27F 16S rRNA primers. Sequences were amplified using an Applied Biosystems™ 7900HT thermocycler. An Agilent® 2100 Bioanalyzer™ capillary electrophoresis unit was used to visualize and separate 16S rRNA sequences from the amplified PCR mixtures. An Applied Biosystems™ 3730 capillary sequencer was used to sequence the 27F forward and 1492R reverse 16S rRNA gene sequences. Forward and reverse sequences were aligned and compared to the NCBI 16S rRNA Nucleotide BLAST® library to identify bacterial species.

Statistical analysis

The effect of adult age and sex on survival of six hours of anoxia was analyzed by logistic regression, with vial as a random effect. To test for age and sex effects on survival of six hour of anoxia using one and twelve-day-old flies from the same parents, a

Fisher's exact test was used. Logistic regression, with vial as a random effect, was used to determine the LD50 values for survival of different durations of anoxia exposure for one and twelve-day-old flies and test the effects of sex and age. ATP data was assessed for heteroscedasticity and normality using the Shapiro-Wilk and Levene's test. The full dataset could not be normalized, so I used generalized linear model (GLM) with an inverse gamma link function to assess the effects of sex, age, and exposure on ATP. Data related to the effects of food change interval and sex on the relative proportion of *Acetobactor* vs. *Lactobacillus* satisfied all the assumptions of the general linear model used for analysis (Peña and Slate, 2006)**.** The effect of food change interval and sex on total bacterial load was analyzed using a generalized linear model with an inverse gamma link function. Logistic regression with eclosion date as a random factor was used to assess the effects of food change interval on six-hour anoxia survival. I used logistic regression with eclosion date as a random factor to assess correlations between bacterial species proportion and anoxia survival in both male and female flies. I used a generalized linear mixed model (GLMM) with eclosion date as a random factor and an inverse gamma link function to assess the effects of total bacterial load and sex on anoxia survival.

All statistical analyses were performed using the program R (R Core Team, 2019). The package lme4 (version 1.1-21) was used for all my generalized linear mixed-effects models (Bates et al., 2015). Generalized linear models (GLM) and general linear models (LM) were created using the built-in R standard library.

Results:

Survival of six hours of anoxia drastically declined over twelve days of age

Approximately 80% of one-day old flies survived six hours of anoxia, but as age increased, survival decreased, and only about 10% of twelve-day-old flies survived (**Fig. 1**). At nearly every age, males had a significantly lower survival than their female counterparts and half the overall survival throughout the entire experiment (**Fig. 1**).

Flies reared from the same parents showed a similar age-effect on anoxia tolerance. Consistent with the initial study, one-day-old flies had an approximate 80% survival rate, while below 20% of twelve-day-old flies survived (**Fig. 2**). Again, males had a lower overall survival than females at twelve-days of age (**Table 2**).

One-day-old flies survived anoxia durations nearly twice as long as twelve-day-old flies

To better characterize the anoxia tolerance of one-day-old and twelve-day-old flies, I tested the effect of anoxia duration on survival. There was a highly significant effect of age (**Table 3**), as six hours of anoxia killed 100% of twelve-day-old flies, while twelve hours of anoxia was required to kill all one-day-old flies (**Fig. 3)**. I calculated the LT50 for each age group using logistic regression, and the LT50 for one-day-old flies was approximately double that of 12-day-old flies (~7.3 hours vs ~3.6 hours). In this experiment, there was not a significant effect of sex on anoxia survival (**Table 3, Fig. 3).**

Age did not affect ATP levels during anoxia

ATP levels fell quickly with time in anoxia, but neither fly age or sex significantly affected ATP (**Table 4, Fig. 4).**

More frequent food change correlated with reduced bacterial content and improved anoxia survival

Based on sequence, gram-staining and cell morphology, *Acetobacter indonesiensis* and *Lactobacillus plantarum* were the only bacteria species present in our *Drosophila*. A higher percentage of the gut bacteria were *Acetobacter* in females compared to males (**Fig. 5a**, Table 5). Although the differences in species composition between males and females appeared to become smaller when food was changed less frequently; there was not a significant interaction between fly age and food change frequency (**Table 5**).

Reducing food change frequency caused an increase in the quantity of bacteria in the guts of 12-day old flies (**Fig. 5b, Table 6**). Reducing food change frequency also reduced the survival of flies in anoxia, regardless of sex (**Fig. 5c, Table 7**). The fraction of bacteria that were *Acetobacter* did not affect anoxia tolerance (**Supplementary Table 1**). A higher quantity of bacteria in the gut predicted a lower survival of anoxia (**Fig. 6, Table 8**). Interestingly, there was also a significant interaction between sex and bacterial load, with males being more negatively affected by increasing bacterial load (**Fig. 6, Table 8**).

Discussion:

Surprisingly, anoxia tolerance declines drastically over the first 12 days of adult *Drosophila* life (**Figs. 1-3**). This decline in anoxia tolerance is not related to the ability to maintain ATP during anoxia (**Fig. 4**). However, decreasing the quantity of bacteria in the gut by frequent food changing improved anoxia tolerance, strongly suggesting that the increasing accumulation of gut bacteria that normally occurs during the first weeks of adult life of *D. melanogaster* (and likely many holometabolous insects) contributes to the age-related decline in anoxia-tolerance (**Figs. 5b and c, 6**). However, I cannot exclude other factors correlated with age and food-change, such as the nutritional value of the food, or negative effects of the accumulation of toxic wastes in unchanged food.

Age-related decline of anoxia tolerance

I found a drastic decline of anoxia tolerance during twelve days of aging past eclosion (**Figs. 1-3**). There was an eight-fold difference in percent survival of six hours of anoxia between one and twelve-day-old flies, averaging the sexes (Figs. 1 and 2). The aging effect on anoxia tolerance was relatively continuous over this age range (Fig. 1), suggesting some relatively continuous underlying process. The aging effect on anoxia tolerance depended strongly on the durations of anoxia were examined (Fig. 3). Both one and twelve-day-old flies had high survival of anoxia durations up to two hours, but with longer durations, survival fell much more quickly for the older flies, and the LT50 fell from 7.3 to 3.6 h.

This strong decline in anoxia tolerance is similar to decreases in upper and lower thermal tolerance observed for *Drosophila* and many adult insects (Bowler and Terblanche, 2008). This decline occurs despite the fact that the mean *Drosophila* lifespan is over 50 days; thus, twelve day old fruit flies are not likely to be appreciably senescent (Samis et al., 1971). The decline of thermal tolerance with age has been shown to be the most dramatic during the first 10 days of adult *Drosophila* life (Bowler and Terblanche, 2008) which further suggests there could be a shared mechanism between anoxia tolerance and thermal tolerance given the steep decline of anoxia tolerance in this study. In thermal tolerance studies, flies transferred to lower temperatures immediately after eclosion tend to be more tolerant to higher temperatures for up to 20 days post-eclosion (Bowler and Terblanche, 2008). Plausibly this could be due to lower temperatures slowing the aging process, or to slowing the accumulation of gut bacteria due to slower feeding rates and the depressing effect of lower temperatures on bacterial population growth. It would be interesting to test whether rearing at lower temperatures would also slow the age-related increases in bacterial abundance and declines in anoxia tolerance. There is also some evidence that immune function declines over this age range in D. melanogaster (see below).

Sex effects on anoxia tolerance

For unclear reasons, males had a much lower overall survival than females in first experiments focusing on survival of six hours of anoxia, but not our second experiment in which I examined various durations of anoxia. (**Figs. 1-3**). Plausibly this related to different genetic lines used in the two experiments since the effect of sex on anoxia

tolerance varies strongly with line (Campbell et al. 2019). Alternatively, differences in rearing conditions (e.g. humidity, food quality) may have altered the effect of sex on anoxia tolerance.

The homogametic sex (females in Diptera) generally live longer in animals (Xirocostas et al., 2020); however, whether the heterogametic sex ages more rapidly is less clear (Lemaître et al., 2020). In *Drosophila* and other insects, females tend to be more tolerant to desiccation, starvation, and heat (Blanckenhorn et al., 2014; Dayal Aggarwal, 2014; Lyons et al., 2014; Millington and Rideout, 2018; Sassi and Hasson, 2013). The mechanisms for such sex-based differences in tolerance are slowly being elucidated. Female *D. melanogaster* tend to have higher concentrations of antioxidants compared to males (Niveditha et al., 2017), which may make them more resistant to the oxidative stress associated with a variety of stresses including recovery from anoxia. Based on differences in survival from exposure to bacterial pathogens by cuticular dusting vs. injection, male *D. melanogaster* appear to suffer a more rapid loss of barrier functions than females (Kubiak and Tinsley, 2017). Anoxia-tolerance of males was be more sensitive to accumulation of gut bacteria (Fig. 6). Conceivably, males may suffer a greater age-related decline in anoxia-tolerance due to a greater degree of immune-related damage as a result of intestinal barrier degradation (Kinross et al., 2009; Rera et al., 2012).

Ability to maintain ATP

The decline in anoxia tolerance with age was not due to a difference in the capacities of one and twelve-day-old flies to maintain ATP. Regardless of age, ATP fell quickly to low levels in anoxia (**Fig. 4**). These results suggest that the variation in anoxic tolerance across 1-12 days of age in *Drosophila* is more likely to be attributed to differences in the rate at which cellular damage occurs or is repaired. In addition to changes in the microbiome, one- and 12-day-old flies may differ in their levels of chaperone proteins, concentration of antioxidants, or tendency for mitochondria to produce ROS during anoxia or reoxygenation (Hochachka, 1986).

Bacterial load and anoxia tolerance

Models explaining anoxia tolerance based on bacterial load had much greater explanatory power than models based on food change (Tables 7, 9) suggesting that increasing bacterial load may be an important mechanism causing the age-related decline in anoxia tolerance. How might this occur? One possibility is that anoxia may allow bacteria to penetrate the gut, leading direct damage to internal tissues and an increased immune response (Kalogeris et al., 2012; Kinross et al., 2009). In mice, intestinal permeability has been shown to increase drastically in anoxia and stimulate immune responses (Souza et al., 2004). Potentially, this could induce inflammation, cellular damage, and death (Kinross et al., 2009). Another possibility is that increased gut bacterial content cause developmental changes that reduce anoxia tolerance, including changes in health of the gut epithelia, or alterations in a variety of stress-resistance

pathways. In the future, it will be important to separate acute from chronic, developmental effects of the bacterial quantity on anoxia tolerance.

Effects of bacterial species

I identified two distinct species of bacteria in the *Drosophila* gut. This result is consistent with the numerous laboratory studies conducted on the *Drosophila* microbiome, as it is common for two to four species to be present (Blum et al., 2013; Broderick et al., 2014; Fink et al., 2013; Wong et al., 2013). Possibly there are additional unculturable bacteria, but multiple studies have found that all bacteria in the *Drosophila* gut can be cultured (Blum et al., 2013; Broderick et al., 2014; Fink et al., 2013; Wong et al., 2013). Variation in the proportion of these bacterial species was not associated with variation in anoxia tolerance, similar to studies that have shown that inflammation of gut epithelia after anoxia is an important mechanism of anoxic damage in mice (Kinross et al. 2009). Overall, our results suggest that future studies of stress-tolerance in animals should examine effects of bacterial load in addition to species composition.

Alternative mechanisms by which the frequency of food change may affect anoxia tolerance

Even though models linking bacterial abundance to anoxia tolerance had much better explanatory power than models based on food change frequency, conceivably, the improvements in anoxia that we observed with more frequent food change could have resulted at least partially from changes in food quality other than bacterial content. It is likely that fly bottles that were changed less frequently accumulated higher

concentrations of nitrogenous waste and other toxins (Belloni et al., 2018); these could have caused the decline in anoxia tolerance with more frequent food changes. While studies have shown that related species, such as *D. suzukii*, may be negatively affected by nitrogenous compounds, *D. melanogaster* are more equip to survive in high-toxin environments since they naturally feed on more densely-populated, rotting fruit (Belloni et al., 2018). More frequent food changes could have also increased the availability of carbohydrates relative to protein available. Daily food changes (and consequent lower gut bacterial content) are associated with shorter lifespan in *D. melanogater*, which has been linked to beneficial effects of the microbiome (Keebaugh et al., 2018). Addition of heat-killed bacteria to sterile media improves lifespan and speeds larval growth of *D. melanogaster*, suggesting that increasing bacterial load, at least up to a point, should improve diet quality by improving protein or vitamin content (Keebaugh et al., 2018). If reduced frequency of food changes increases the protein content of the diet, this has been shown to reduce the hypoxia tolerance of *D. melanogaster* (Vigne and Frelin, 2010). Further tests to separate effects of bacterial content from other aspects of diet quality will be necessary to confirm whether increasing bacterial content is indeed a causal mechanism for the age-related decline in anoxia tolerance.

Other age-related declines in stress tolerance

As pointed out by Bowler and Terblanch (2008), age-related variation in stress tolerance is common among insects but very poorly understood mechanistically. Conceivably, there could be a progressive transfer of proteins used for stress-resistance such as heat shock proteins toward reproductive functions with age. There is a general

trend for animals to have reduced immune function with age (Gardner, 1980). Newly emerged damselflies have more hemocytes, but lower phenoloxidase activities than sexually mature animals (Rolff, 2001). Similarly, hemocyte numbers decline over the first two weeks of age in honey bees, while phenoloxidase activities rise (Schmid et al., 2008). Immune responses have been reported to decline as *D. melanogaster* age from one to four weeks (Kubiak and Tinsley, 2017), with a concurrent decline in the number of phagocytizing hemocytes (Mackenzie et al., 2011). Melanization reaction against filarial worms is lower in 14 day-old than one-day-old mosquitos (Christensen et al., 1986). Together these data suggest that increasing age may be associated with a general transfer of investments from stress-resistance to reproduction well before senescence occurs. A reduced immune capacity in older adults may also synergize with higher bacterial loads, leading to more damage or inflammation associated with bacterial introgression into or through the anoxic gut, contributing to the observed age-related decline in anoxia tolerance.

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APPENDIX A

DATA COLLECTED AUGUST 2014 – NOVEMBER 2016

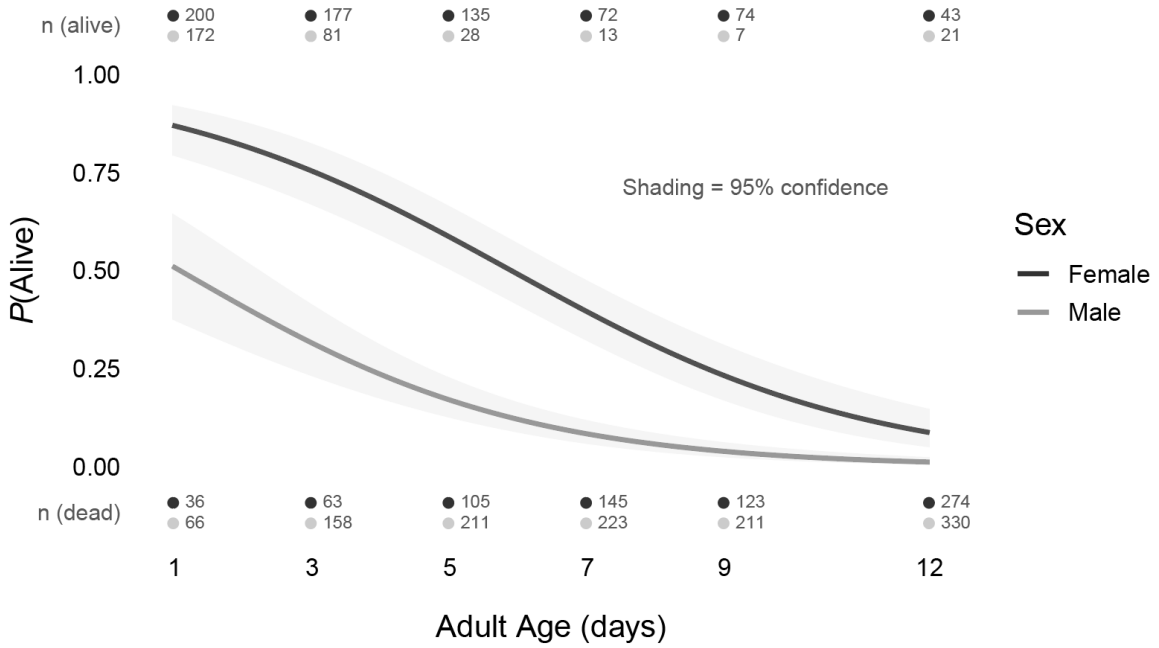


Fig. 1 The effect of fly age (1-12 days) on survival of six hours of anoxia. Anoxia survival declined significantly with age. Females had more than double the overall survival of males. In this and subsequent figures, means and 95% confidence intervals shown, and sample sizes and number of vials for each age group are indicated). Results of generalized linear mixed model analysis found in **Table 1**.

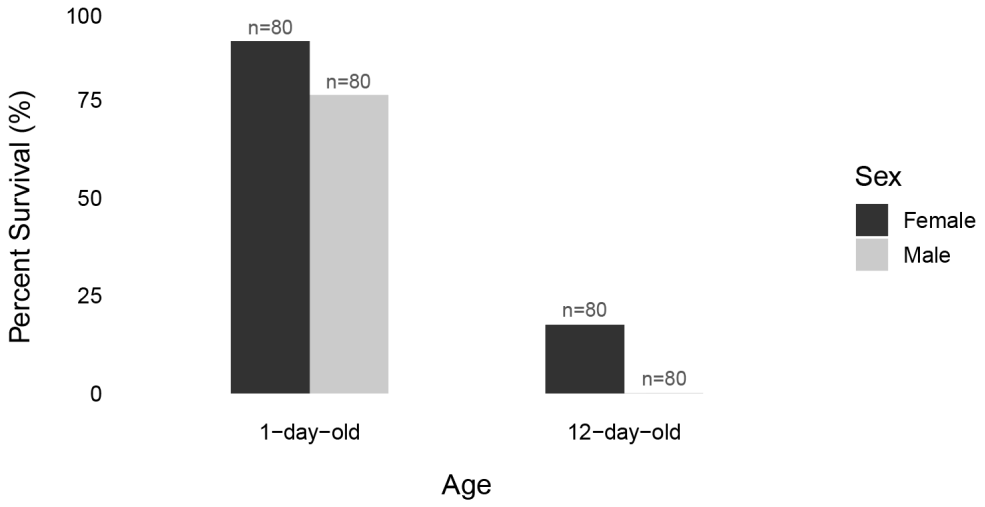


Fig. 2 Effect of fly age on survival of six hours of anoxia for flies from a single population. Twelve-day-old flies had lower survival than one day old flies. Females had higher overall survival. Results of Fisher's Exact test found in **Table 2**.

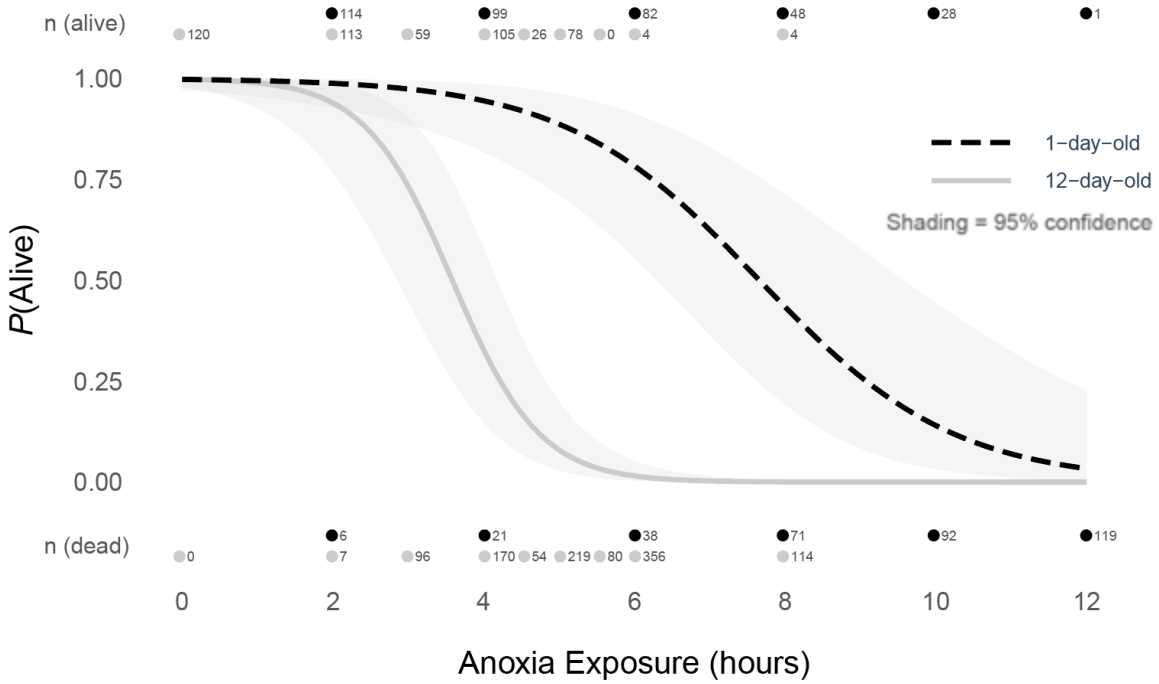
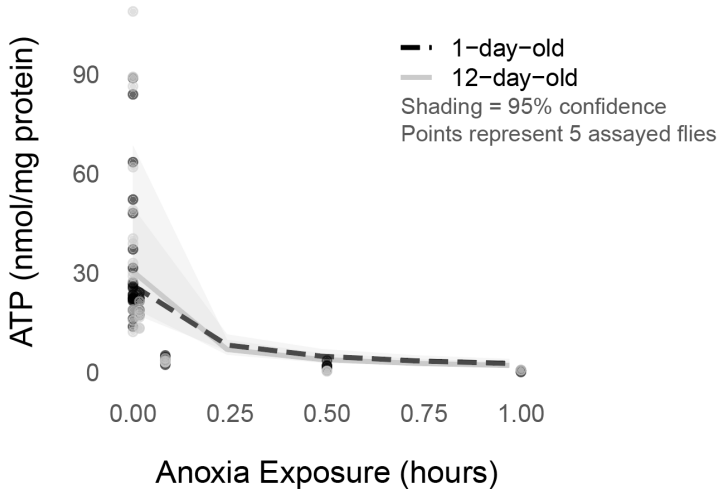


Fig 3 The effect of anoxia duration on survival of one and twelve-day-old flies. Points represent average survival for age groups and sex following different durations of anoxia exposure. The labeled dots between 4 and 6 represent 4.5, 5, and 5.5 hour timepoints. *P(alive)* represents probability of survival. Sexes were pooled because sex did not significantly affect survival. At two hours of anoxia and less, age did not affect survival, but at all other durations, the younger flies had significantly higher survival. Overall, there was a significant effect of age on survival. Results of generalized linear mixed model analysis found in **Table 3**.

A.



B.

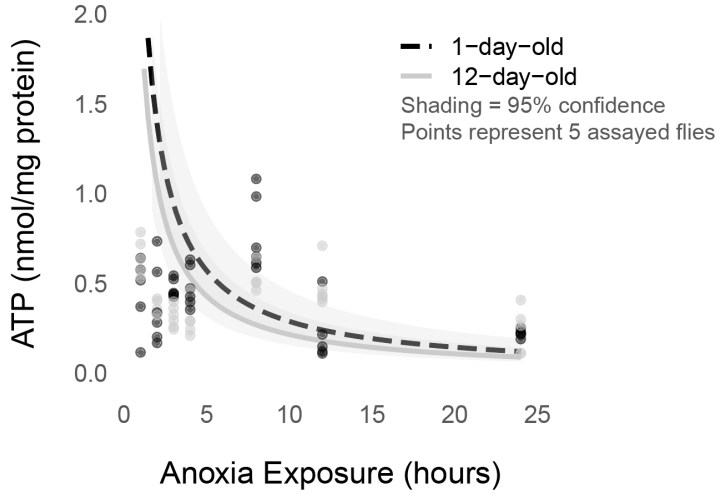


Fig 4 Effect of duration of anoxia on ATP levels in one and twelve-day-old flies. Semi-transparent shading represents 95% confidence intervals with overlap between age groups appearing darker. Both panels were from the same experiment with panel (A) showing a closer view of shorter anoxia durations and panel (B) showing all the anoxia timepoints (0 through 24 hours). There was no significant effect of age on ATP levels at any exposure duration or overall, nor was there an effect of sex on ATP levels with an exception occurring at three hours most-likely attributed to experimental error. ATP declined significantly with time. Six samples of thirty adults were assayed for each data point. Results of generalized linear mixed model analysis found in **Table 4**.

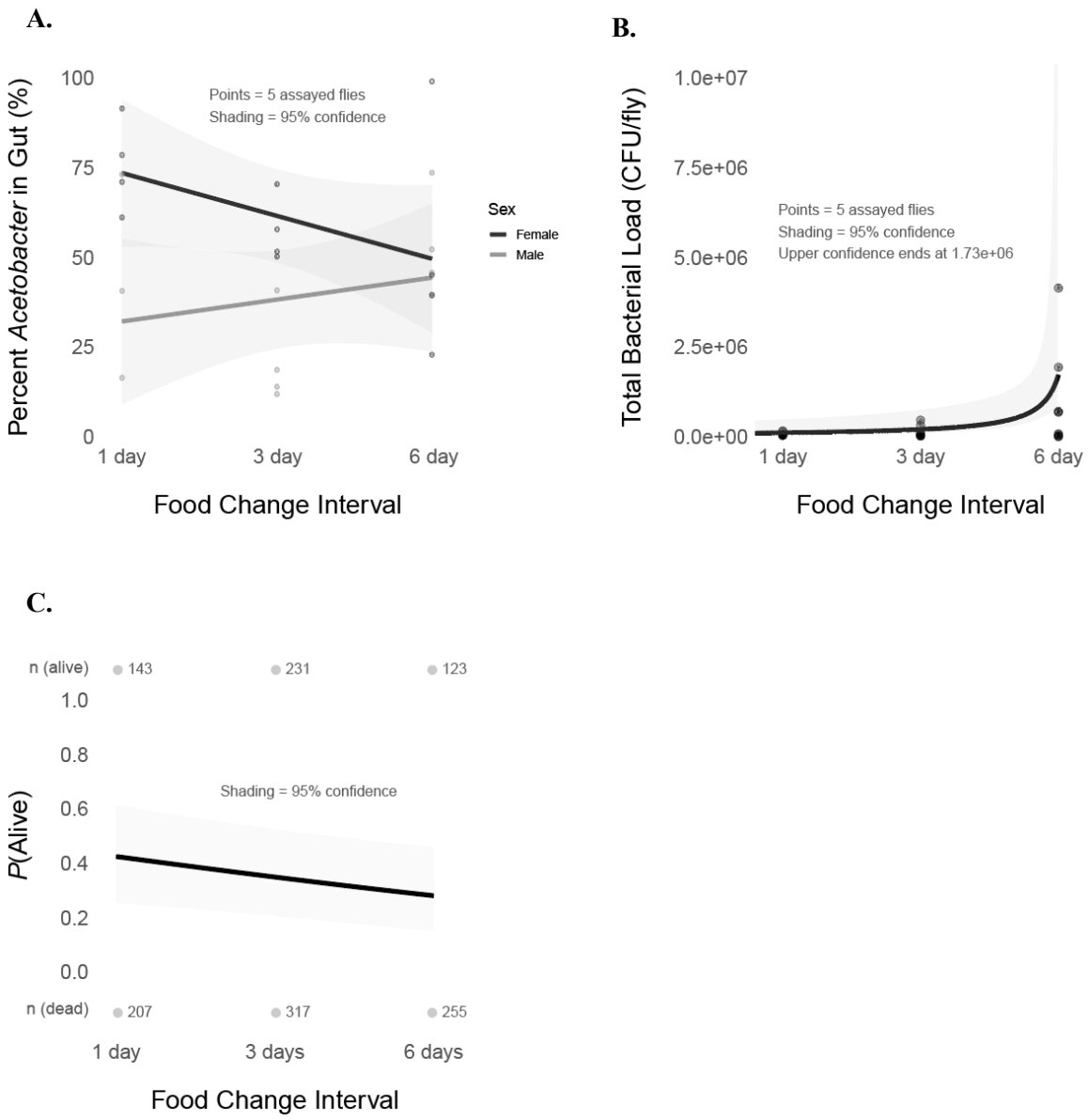


Fig 5 A. Sex significantly affected the percentage of *Acetobacter* in the gut (**Table 5**).
Fig 5 B. Reducing food change frequency was associated with a higher bacterial load in the guts of flies (**Table 6**).
Fig 5 C. Reducing food change frequency was associated with a significant decline in survival after six hours of anoxia (**Table 7**). *P(alive)* represents probability of survival.

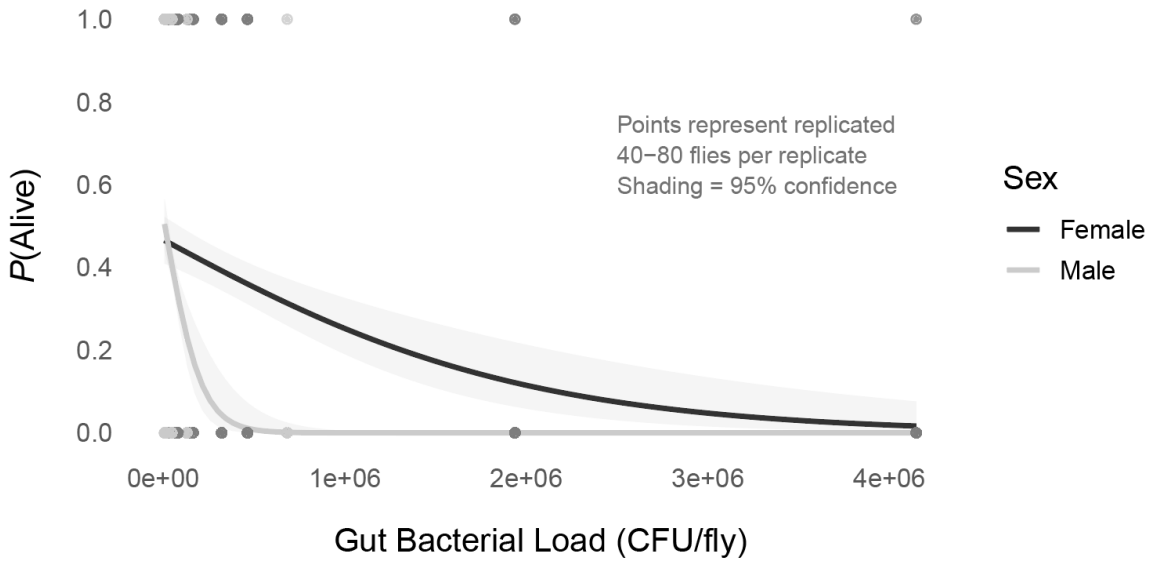


Fig 6. A higher quantity of bacteria in the gut was associated with a lower percent survival; and there was a significant interaction effects with males more negatively impacted by high bacterial loads than females (**Table 8**). *P(alive)* represents probability of survival.

APPENDIX B

DATA COLLECTED AUGUST 2014 – NOVEMBER 2016

Table 1 Generalized linear mixed model (GLMM) for the effects of age and sex on fly survival of six-hours of anoxia

Variable	*Estimate*	*SE*	*Z*	*P*
(Intercept)	2.2864	0.3187	7.1745	7.26e-13 ***
Age	-0.3861	0.044	-8.7645	< 2e-16 ***
Sex	-1.8384	0.4528	-4.0602	4.90e-05 ***
Age × Sex	-0.0179	0.0645	-0.2779	0.7811

Random effect(s)	*Variance*	*SD*	*Observations*
Group: Vial	1.506	1.227	153

AIC	381.4114
BIC	396.5636
Log-Likelihood	-185.7
Deviance	371.4
Theoretical $R^2_{GLMM(m)}$	0.41
Theoretical $R^2_{GLMM(c)}$	0.5951
Delta $R^2_{GLMM(m)}$	0.3595
Delta $R^2_{GLMM(c)}$	0.5221
DF	148

Note:
a. Dependent variable: alive survival
b. Family: bionomial (link=logit)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion
* P<0.05; ** P<0.01; *** P<0.001

Table 2 Fisher's Exact Test for the effects of age and sex on six-hour anoxia survival of adults from the same parents

Variable		**Alive**	**Dead**
Sex	*n=320*		
	Female	86	74
	Male	61	99
	Total	147	173
	Odds ratio	1.882	
	P	0.007***	
Age	*n=320*		
	One-day-old	133	27
	Twelve-day-old	14	146
	Total	147	173
	Odds ratio	50.239	
	P	< 2.2e-16***	

Note:
* *P*<0.05; ** *P*<0.01; *** *P*<0.001

Table 3 Generalized linear mixed model (GLMM) for the effects of age, sex, and anoxia exposure duration on fly survival

Variable	*Estimate*	*SE*	*Z*	*P*
(Intercept)	5.9062	1.3107	4.5062	6.6e-06 ***
Age	0.0232	0.1333	0.1743	0.8616
Sex	0.21	1.2456	0.1686	0.8661
Exposure	-0.6879	0.1821	-3.7765	0.0002***
Age × Exposure	-0.0865	0.0238	-3.6341	0.0003***
Sex × Exposure	-0.1747	0.2112	-0.8275	0.408

Random effect(s)	*Variance*	*SD*	*Observations*
Group: Vial	5.699	2.387	117

AIC	310.3427
BIC	329.6779
Log-Likelihood	-148.2
Deviance	296.3
Theoretical $R^2_{GLMM(m)}$	0.5832
Theoretical $R^2_{GLMM(c)}$	0.8475
Delta $R^2_{GLMM(m)}$	0.5314
Delta $R^2_{GLMM(c)}$	0.7722
DF	110

Note:
a. Dependent variable: alive survival
b. Family: bionomial (link=logit)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion
* P<0.05; ** P<0.01; *** P<0.001

Table 4 Generalized linear model (GLM) for the effects of age, sex, and anoxia exposure duration on ATP levels

Variable	*Estimate*	*SE*	*T*	*P*
(Intercept)	0.0385	0.0099	3.8763	0.0002***
Age	-0.0005	0.0012	-0.4019	0.6883
Sex	-0.0054	0.0128	-0.4183	0.6764
Exposure	0.3316	0.0709	4.678	6.54e-06 ***
Age × Sex	-0.0002	0.0015	-0.1295	0.8971
Age × Exposure	0.0107	0.0097	1.1013	0.2726
Sex × Exposure	0.1696	0.1211	1.4003	0.1635
Age × Sex × Exposure	-0.0222	0.0141	-1.5769	0.117

AIC	635.91
BIC	663.24
Null deviance	549.13 (*DF*=153)
Residual deviance	113.14 (*DF*=153)
McFadden's pseudo-R^2	0.794

Note:
a. Dependent variable: ATP concentration
b. Family: Gamma (inverse, φ = 0.7498)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion; φ, Gamma dispersion parameter
* P<0.05; ** P<0.01; *** P<0.001

Table 5 General linear model (LM) for the effects of sex and food change interval on the proportion of *Acetobacter* vs. *Lactobacillus*

Variable	*Estimate*	*SE*	*F*	*P*
(Intercept)	0.8516	0.1651	5.1581	0.0001***
Sex	-0.5998	0.2471	-2.427	0.0253*
Treatment	-0.1206	0.0764	-1.5784	0.131
Sex × Treatment	0.1823	0.1123	1.6234	0.121

AIC	0.4188
Residual SE	0.2162 (*DF*=19)
R^2	0.3341
Adj. R^2	0.2289
F(3,19)	3.18
P	0.04775

Assumptions (GVLMA)	*Value*	*P*
Global Stat	5.3842	0.2501
Skewness	3.1401	0.07639
Kurtosis	0.1555	0.69333
Link Function	1.0631	0.3025
Heteroscedasticity	1.0254	0.31124

Note:
a. Dependent variable: Percent *Acetobactor* (relative to total load)
b. Type: OLS linear regression
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; GVLMA, Global Validation of Linear Models
* P<0.05; ** P<0.01; *** P<0.001

Table 6 Generalized linear model (GLM) for the effects of bottle transfer interval and sex on gut bacterial load

Variable	*Estimate*	*SE*	*T*	*P*
(Intercept)	1.36E-05	5.12E-06	2.6494	0.0158
Food change	-4.32E-06	1.71E-06	-2.5222	0.0207*
Sex	1.65E-05	1.54E-05	1.0735	0.2965
Food change × Sex	-3.84E-06	5.39E-06	-0.7128	0.4846

AIC	612.5738
BIC	618.2513
Null deviance	64.523 (*DF*=22)
Residual deviance	26.882 (*DF*=19)
McFadden's pseudo-R^2	0.5833693

Note:
a. Dependent variable: Gut bacterial load
b. Family: Gamma (inverse, φ = 1.025)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion; φ, Gamma dispersion parameter
* P<0.05; ** P<0.01; *** P<0.001

Table 7 Generalized linear mixed model (GLMM) for the effects of food change on survival

Variable	*Estimate*	*SE*	*Z*	*P*
(Intercept)	0.0156	0.4561	0.0342	0.9727
Sex	-0.2005	0.3601	-0.5567	0.5777
Food change	-0.3195	0.1407	-2.2706	0.0232*
Food change × Sex	0.2232	0.1731	1.2894	0.1973

Random effect(s)	*Variance*	*SD*	*Observations*
Group: Eclosion day	0.4932	0.7023	4

AIC	195.9146
BIC	201.5921
Log-Likelihood	-93
Deviance	185.9
Theoretical $R^2_{GLMM(m)}$	0.01244938
Theoretical $R^2_{GLMM(c)}$	0.1411962
Delta $R^2_{GLMM(m)}$	0.0101009
Delta $R^2_{GLMM(c)}$	0.1145606
DF	18

Note:
a. Dependent variable: alive survival
b. Family: bionomial (link=logit)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion
* P<0.05; ** P<0.01; *** P<0.001

Table 8 Generalized linear mixed models (GLMM) for the effects of species proportion on anoxia survival

Variable	*Estimate*	*SE*	*Z*	*P*
Male flies:				
(Intercept)	-0.2677	0.5061	-0.529	0.5968
Acetobacter /total load	-0.7469	0.5663	-1.3189	0.1872
Random effect(s)	*Variance*	*SD*	*Observations*	*Groups*
Group: Eclosion day	0.8362	0.9145	11	4
AIC	111			
BIC	112.2			
Log-Likelihood	-52.5			
Deviance	105			
Theoretical $R^2_{GLMM(m)}$	0.006659782			
Theoretical $R^2_{GLMM(c)}$	0.2079798			
Delta $R^2_{GLMM(m)}$	0.005472422			
Delta $R^2_{GLMM(c)}$	0.1708994			
DF	8			
Female flies:				
Variable	*Estimate*	*SE*	*Z*	*P*
(Intercept)	0.0916	0.4944	0.1853	0.853
Acetobacter /total load	-1.0849	0.6836	-1.5872	0.1125
Random effect(s)	*Variance*	*SD*	*Observations*	*Groups*
Group: Eclosion day	0.2409	0.4908	11	4
AIC	46.9			
BIC	48.1			
Log-Likelihood	-20.4			
Deviance	40.9			
Theoretical $R^2_{GLMM(m)}$	0.012140612			
Theoretical $R^2_{GLMM(c)}$	0.07952893			
Delta $R^2_{GLMM(m)}$	0.009461661			
Delta $R^2_{GLMM(c)}$	0.06198005			
DF	8			

Note:
a. Dependent variable: alive survival
b. Family: bionomial (link=logit)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion
* P<0.05; ** P<0.01; *** P<0.001

Table 9 Generalized linear model (GLM,logistic regression) for the effects of bacterial load on anoxia survival

Variable	*Estimate*	*SE*	*T*	*P*
(Intercept)	-0.1318	0.1157	-1.1386	0.2549
Total bacterial load	-9.543e-07	2.067e-07	-4.6172	3.89e-06***
Sex	0.2283	0.1813	1.259	0.208
Total bacterial load × Sex	-8.948e-06	2.401e-06	-3.7276	0.0002***

AIC	246.9052
BIC	251.2694
Null deviance	261.72 (*DF*=21)
Residual deviance	151.56 (*DF*=18)
McFadden's pseudo-R^2	0.4209014

Note:
a. Dependent variable: alive survival
b. Family: bionomial (link=logit)
c. SE, standard error; SD, standard deviation; DF, degrees of freedom; AIC, Akaike information criterion; BIC, Bayesian information criterion
* P<0.05; ** P<0.01; *** P<0.001