Drosophila development, physiology, behaviour, and lifespan are influenced by altered dietary composition.

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7 figures, 4 tables

Keywords: diet, *D. melanogaster*, thermal preference, geotaxis, learning, memory, morphology, locomotion, muscle, bioactive peptides

Abbreviations: MCH: 4-methylcyclohexane, OCT- 3-octanol, monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG).

Published in FLY March 2017.

http://dx.doi.org/10.1080/19336934.2017.1304331

http://www.tandfonline.com/doi/full/10.1080/19336934.2017.1304331

Abstract

Diet profoundly influences the behaviour of animals across many phyla. Despite this, most laboratories employing model organisms, such as *Drosophila*, use multiple, different, commercial or custom-made media for rearing their animals. In addition to measuring growth, fecundity and longevity, we employed several behavioural and physiological assays to determine if and how altering food media influence wild-type (Canton S) *Drosophila melanogaster*, at larval, pupal, and adult stages. Comparing two commonly used commercial food media we observed several key developmental and morphological differences. Third-instar larvae and pupae developmental timing, body weight and size, and even lifespan significantly differed between the two diets, and some of these differences persisted into adulthood. Diet was also found to produce significantly different thermal preference, locomotory capacity for geotaxis, feeding rates, and lower muscle response to hormonal stimulation. There were no differences, however, in adult thermal preferences, in the number or viability of eggs laid, or in olfactory learning and memory between the diets. We characterized the composition of the two diets and found particularly significant differences in cholesterol and (phospho)lipids between them. Notably, diacylglycerol (DAG) concentrations vary substantially between the two diets, and may contribute to key phenotypic differences, including lifespan. Overall, the data confirm that two different diets can profoundly influence the behaviour, physiology, morphology and development of wild-type *Drosophila,* with greater behavioural and physiological differences occurring during the larval stages.

Introduction

The fruit fly, *Drosophila melanogaster*, is a focal model organism for investigations of almost all aspects of organismal biology. Since the origins of *Drosophila* research, investigators have employed varied media upon which to rear flies; the ability of flies to adapt and survive on a wide-variety of food sources has likely promoted *Drosophila* divergence and is at least one of their attractive features as a model organism. Since the beginning of the 20th century, a great number of investigations have sought to determine the optimum rearing conditions and media for maximizing the growth and development of *Drosophila* [1,2]. Subsequently, it has become common practice to alter one or two constituents of commonly used media in order to ascertain their role in various physiological, developmental or behavioural paradigms [3–6]. However, few investigations have directly addressed potential confounding effects of using different rearing media and what this might mean in terms of phenotypic differences.

Here we assessed two commercially available *Drosophila* diets to test for dietary derived differences in development, physiology, morphology or behaviour. Numerous experimental approaches exist for the examination, differentiation and characterization of dietary effects. Timing of development to landmark stages such as instars, pupation and eclosion, is perhaps the simplest measure of effect [7]. Measures of fitness, reproductive rates and longevity are also key indicators [8–12]. It is also common to examine the effects of diet or other factors on internal and external structural development, and general morphology [1,13–15]. A number of metrics can be used to examine morphological differences that persist across all stages of development; the two most heavily investigated stages of *Drosophila* development are the larval and adult forms. Most

77 commonly, weight, length and width are used when examining larvae [16]. In adults, the number of
78 phenotypic interest points is substantially higher due to their use as correlates to genotypic
79 markers (i.e. genetics / functional genomics). However, when examining changes in gross
80 morphology, previous research has typically focussed on body size, wing length, colour, and
81 weight [16–18].

82 Behavioural assays have been developed to the point that some assessments can begin as
83 early as the embryo (e.g. peristalsis [19]). Numerous assays have been designed to study larval
84 behaviour including gustation [20], olfaction [21], vision [22], foraging [23], locomotion [24–27], as well as
85 thermotactic-[28,29] and phototactic-behaviour [30,31]. For adults, assays include thermotatic
86 behaviour [32,33], geotaxis [34], learning and memory [35], social interactions [36], aggression [37],
87 courtship [38], vision [39], acoustic activity [40], olfaction[41], taste[42], and others [43].

88 Whereas many diet related studies tend to focus on only a single phenotypic criterion for
89 evaluation, here we took a broader yet more integrative approach, making use of several well-
90 established *Drosophila* research 'tools' to test the hypothesis that development, morphology,
91 physiology, and/or behaviour, at larval, pupal, and adult stages, differ depending upon the
92 rearing media used. The breadth of analyses was chosen so as to capture as wide a range of
93 potential phenotypic changes associated with diet as possible. We reared *D. melanogaster*
94 Canton S, in parallel, on either of two commercial diets: Formula 4-24 ® media or Jazz mix ®.
95 We examined the following: (i) developmental timing of larva, pupae and adults, (ii) weight and
96 size of third-instar larvae and size of adults, (iii) effects of a stress hormone on third-instar body-
97 wall muscle force production, and (iv) a number of larval and adult behaviours including thermal
98 preference, response to a temperature extreme, velocity of larval crawling, locomotory response
99 to gravity (negative geotaxis), and capacity for learning and memory in adults. We sought to

initially relate observed differences and similarities to the composition of both diets by assessing the fat, carbohydrate, protein, ash, and moisture content of each medium. More detailed analyses revealed significant differences in phospholipid concentrations in the starting dietary material which translated to substantive differences in the larval and adult tissues. Our results confirm significant physical and behavioural phenotypic differences that occur as a consequence of using different dietary materials.

Materials and Methods

Fly stocks

All flies used were *D. melanogaster* Canton S, obtained from the Bloomington Drosophila stock center (Bloomington, IN, USA). Flies were kept at constant temperature (21°C) and humidity (65-70%), under a 12:12h light:dark cycle. Fly vials were randomised in the stock trays across all experiments and treatments. All flies we maintained on their respective dietary media for a minimum of 4 generations prior to testing. Flies were reared using either Formula 4-24 ® media (Carolina Biological) or Jazz mix ® (Fisher Scientific), in VWR ® polypropylene 25mm x 95mm *Drosophila* vials. The composition of these diets, listed by the manufacturer is as follows: Formula 4-24 – Oat flour, soy flour, wheat flour, other starches, dibasic calcium phosphate, calcium carbonate, citric acid, niocinamide, riboflavin, sodium chloride, sodium iron pyrophosphate, sucrose, thiamine, mononitrate, brewer's yeast, emulsifier preservatives, mold inhibitor, food colouring; Jazz-mix – sugar, corn meal, yeast, agar, benzoic acid, methyl paraben, propionic acid. The Formula 4-24 diet required separate application of yeast pellets (*Saccharomyces cerevisiae*) and saturation of this dry media mixture with water. The Jazz-mix did not require yeast pellets as yeast is contained within the media; however, each final vial of media required that the stock food be mixed with 10 mL of water, and boiled for 10 minutes. Dietary effects on rearing for the two groups were assessed contemporaneously under all conditions. Other than the rearing media, all other conditions were identical for all flies used. A control diet was used for comparison in some experiments. The control diet is the media used at

Massachusetts Institute of Technology and is composed of: distilled water, agar, brewer's yeast, corn meal, granulated sugar, and methyl 4 hydroxybenzoate dissolved in ethyl alcohol.

Growth and development

To assess if either diet had an effect on the timing of landmark developmental stages (i.e. third-instar, and pupation), we placed five females 5 days post eclosion from each diet and placed them for 2 hours on grape agar dishes with yeast paste added. 25 eggs were then transferred to vials containing fresh media from each of the diets. Timing to each of the developmental stages was then assessed in each of the 10 replicate vials.

In a separate experiment, we determined the longevity on each of the diets by putting 50 flies of each sex (the day of eclosion) reared on the separate diets into separate vials containing the same diet they were reared on (Jazz-mix or Formula 4-24). Each day we counted and removed any dead adults. We transferred the adults onto fresh media every 7 days. We repeated these procedures until all flies had died in each of the 8-10 replicate vials tested for each diet.

To determine if diet has an effect on the total number of eggs laid by females, 5 adult females 5 days post-eclosion were obtained from each of the respective rearing media and transferred onto a grape-agar plate with fresh yeast paste added. The females were kept at 25 degrees Celsius for 3 days in an incubator. Each day at the 24 hour point, fresh grape-agar plates with yeast paste were exchanged for the day old plates, and the total number of eggs laid was counted. This was repeated 10 times for each of the 3 diets. We transferred 100 eggs from each of plates to a fresh grape-agar plate with yeast paste kept at 25 degrees Celsius and subsequently counted to number of eggs that progressed to first-instar larvae over five-days. This was repeated 10 times for each of the 3 diets. Next, one virgin male and one virgin female were transferred to a fresh vial of food from each of the diets. After 4 days, the 2 adults were transferred to fresh

grape-agar with yeast paste for 24 hours and kept at 25 degrees Celsius. 50 eggs were isolated the following day, transferred to a fresh agar plate, and the total number of eggs that progressed to first-instar larvae was assessed. This procedure was repeated 7 times for each of the diets.

To examine if either of the commercial rearing media affected the morphology of *Drosophila*, the weight and size of wandering third-instar larvae and the size of adults reared on each of the diets were measured. To assess the 'wet' larval weight we pooled 10 randomly selected larvae from each diet and weighed them collectively on a Mettler Toledo mx5 scale; these larvae were then kept in a 60°C oven overnight, and the resulting 'dry' weight measured. This procedure was repeated for 10 sets (i.e. separate stock vials) of larvae on each diet. To examine if these diets affected body size, the length and width of third-instar larvae (N=40 for length, N=50 for width) reared on either diet were measured. As the length of third-instar larvae can vary depending on the contractile state of the bodywall muscles, the larvae were pinned at one end and stretched during size measurements. For metrics of adult *Drosophila*, it has been previously established that wing-length is strongly correlated with adult size; to test for an effect of diet, the length of the 4th longitudinal vein was measured[17].

Muscle contractions

To determine if changes in size as a consequence of differential dietary rearing had physiological implications, we measured changes in octopamine-induced muscle contractions in larvae reared on both diets. Octopamine is a neurotransmitter and neuromodulator in invertebrates (Roeder, 2005) and is present in the *Drosophila* nervous system[44]. Octopamine induces slow contractions (lasting up to 5 min) in *Drosophila* larval body wall muscles which are easily detected as an increase in muscle tonus[45]. Detailed descriptions of the larval third-instar dissection and HL6 composition are reported elsewhere[46]. Briefly, wandering third-instar larvae

were pinned longitudinally on a magnetic dissection dish. A superficial incision was made along the dorsal mid-line, the larva eviscerated and the CNS removed[47]. After the dissection, the anterior pin was removed, and the larva was hooked to a Grass FT03 tension transducer (Grass Instruments, Quincy, MA) so that contractions of the longitudinal muscles were parallel to the movement of the force transducer spring. The longitudinal muscles were stretched slightly by pulling the body in a dorsal and anterior direction. Contractions were amplified using a MOD CP 122A amplifier (Grass Telefactor, W. Warwick, RI) and were recorded using a computerized data acquisition system. The recording dish had a volume of approximately 0.2-0.4 mL and was perfused continuously at a rate of 0.7 mL/min. Excess fluid was removed by continuous suction.

Larval thermal preference

To assess thermal preference, a linear thermal gradient of 15°C to 30°C was generated using an aluminum dish machined into a rectangular lane (48mm x 20mm x 9 mm), which was placed on top of two Peltier units, each positioned at either end of the lane. Each Peltier unit had an independent digitally controlled power supply box (Brock University Electronics Division), ensuring accurate and constant temperature extremes. The temperature range and linear nature of the gradient were confirmed using a thermal camera (FLIR SC660, FLIR Systems, Inc.), connected to a computerized data acquisition system (Examine-R, FLIR Systems, Inc.). During experiments, the thermal gradient was monitored using two thermocouples, one at each end of the lane. Third-instar larvae were removed from stock vials (maintained at 21°C) without anaesthesia, washed in dH_20 (i.e. to avoid contaminating the testing surface with food) and placed in the center of the lane. Testing was in the dark, and images were captured under IR light using a Vario-Sonnar Super Steady Shot© camcorder (Sony Carl Zeiss); the video stream was captured to still images every second using HandyAvi software (AZcendant Software, Tempe,

AZ). ImageJ (NIH) software was used to assess the final positional co-ordinates and the corresponding temperature for each larva after 10 minutes, and this was scored as a thermal preference. The sample size for these experiments was 20 trials per diet (each trial containing 20 larvae). Prior to experimentation any potential side or wall bias was assessed by examining larval distribution in the lane set ubiquitously at 22°C. We arbitrarily divided the lane into four equal quadrants and observed no statistical differences between them, nor were there any side or wall biases.

Larval heat avoidance response

A modified thermal gradient (22-38°C) was established by setting the Peltier units to extreme temperatures. Using the same approach as described above, we placed larvae (N=4 per trial) at the hot end (38°C) of the lane and assessed their movement within the gradient for two minutes, acquiring still images every second. Using Image J (NIH), larval position after two minutes was assessed as total displacement, measured in centimeters. The sample size for warm avoidance was 54 trials per diet. In an alternate set of experiments, larvae were placed on the cold side (22°C) of the lane to test for any preference for the warmer temperatures. The sample size when placing larvae on the cool side was 10 trials per diet.

Larval Velocity

Drosophila larvae are well known to have negative phototaxis[31], so we placed larvae at one end of a rectangular lane (to mitigate escape and to encourage locomotion in a linear direction) set to a constant 21°C, then using fiber-optic cables, cast light directly onto the larvae, which caused them to crawl away. We recorded their behaviour on a video camcorder (see

229 above) and calculated their velocity over a distance of 4 mm. Calculations of larval velocity were
230 determined for 10 individual larvae from each of the two diets.

231 *Adult Negative Geotaxis*

232 Adult flies were subjected to a negative geotaxis assay in an apparatus previously
233 described[34] to assess speed and propensity to climb. Flies used for this study were between one
234 and eight days post-eclosion. Using CO_2 anesthesia, flies were removed from their stock vials
235 one day prior to testing, and placed in groups of 25 in separate clean vials containing the food on
236 which they were reared. The following day, we placed the flies into each of six testing chambers
237 and performed the negative geotaxis assay. The apparatus containing the six vials was rapidly
238 descended vertically from a height of 73 mm. We subsequently counted the number of flies that
239 ascended more than 45mm (50% of the vial height) after 4 seconds, and after 55 seconds. The
240 assay was repeated three times for each group at 1 minute intervals, to establish the mean per
241 experiment. For each diet, 72 vials (each containing 25 flies) were assessed (i.e. 1800 flies/diet).

242 *Adult thermotaxis*

243 To test for thermal preference in adult flies we followed a previously established protocol
244 from Hong et al. (2006)[48]. Briefly, we placed flies in a glass tube (0.6cm x 42cm) fitted with a
245 copper jacket. Either end of the copper jacket was connected to a digitally controlled Peltier
246 device (Thermal Cycler; Brock University Electronics Division). The temperature gradient along
247 the glass tube was linear and ranged from 14°C to 36°C, with a slope of (1.9°C/cm). The gradient
248 was confirmed using a thermal camera (FLIR SC660, FLIR Systems, Inc.) connected to an
249 acquisition program (Examine-R, FLIR Systems, Inc.). All flies used during these experiments
250 were less than 12 days post-eclosion. Thirty flies were placed within the tube at a time and were

allowed to distribute within the gradient for 25 minutes in the dark before a photograph was taken with a digital camera. We assigned each fly a thermal preference value based upon its location in the tube and the temperature value generated by the thermal camera. Sample size for these experiments was 19 trials for Formula 4-24 and 17 trials for Jazz mix.

Adult Learning and Memory (olfactory operant conditioning assay)

To assess if the two commercial rearing media influenced *Drosophila* learning and memory, we subjected adults to a modified version of the Tully and Quinn (1985)[35] T-Maze, which examines olfactory-based associative learning and memory. Flies (75-100 at a time), between one and eight days post-eclosion, were placed within a copper-grid wire lined acrylic chamber through which air and odours could flow. Flies were classically conditioned to associate one of two odours, 4-Methylcyclohexanol (MCH; Aldrich – 101123210) or 3-Octanol (OCT; Sigma-Aldrich – W358126), with a 90V shock as the negative stimulus. The odours were produced by dissolving these chemicals in mineral oil at a 10^{-6} dilution. Compressed air was forced through the mineral oil at a constant flow rate (500 mL/min), and the odour stream was diverted to appropriate test chambers. A no odour condition was produced simply by bubbling air through mineral oil. Half of the flies were trained to associate the shock with MCH, and the other half were trained to associate the shock with OCT. Each training session consisted of 90 s with air, 60 s with shock and odour #1, 30 s with air, 60 s with odour #2, and 30 s with air. After training, the flies were transferred down an elevator to a point where they were able to choose between two collection tubes, one containing odour #1 and the other with odour #2. The performance of the flies was calculated as in Tully and Quinn (1985)[35]. A learning index was determined as the fraction of flies that avoided the shock-associated odour minus the fraction of flies that preferred the shocked-associated odour. Since we trained flies to associate shock with

either odour, we averaged the two odour-shock groups for each of the diets. If all avoided the shock-associated odour (perfect learning), the index value would be 1; if all preferred the shock-associated odour, the index value would be -1. A learning index value was computed for each group 2, 5 and 10 minutes after training. Ten independent groups of 75-100 flies were assessed for each diet.

Adult feeding assay

The capillary feeding (CAFE) assay was used to assay rates of food consumption between adults reared on different diets. The CAFE assay consists of a 50mL conical centrifuge tube with 5mL of distilled water to maintain humidity. A clean, empty *Drosophila* vial with a sponge top was placed inside the conical tube. A small hole is melted into the top of the conical tube and the underlying sponge. A 5µL calibrated glass tube (Accu-fill 90, micropet) was inserted into the hole, roughly 1cm past the sponge. The glass tube was filled with 5µL of a solution of 5% sucrose and 5% yeast extract. Four males or four females were transferred from their respective rearing diets, into the assay for 8 hours and kept in an incubator at 25 degrees Celsius. The total amount of food consumed over the 8 hours was assessed by directly examining the calibrated glass. This was repeated 12 times for each of the diets.

Composition of media and fly tissue

Initially, to examine the compositional differences between the two commercially available diets, we submitted samples of each to a regulatory agency laboratory for the analysis of moisture, protein, ash, fat and carbohydrate (Agri-Food laboratories, Guelph, Ontario, Canada). Next, we assessed the lipid composition of each diet, as well as whole *Drosophila* tissue(5 animals per replicate, snap frozen in liquid nitrogen at the beginning of each

296 developmental stage assessed: 3rd instar, pupal and adult). Drosophila tissue samples were
297 manually homogenized on ice in 1x phosphate buffered saline. Samples were then subjected to
298 lipid/protein extraction using the Bligh and Dyer method[49] with some modifications[50]; the
299 organic phase was subsequently dried under nitrogen and stored at - 30 °C prior to lipid analysis,
300 and the combined interphase and aqueous phase was used to estimate protein concentration.
301 Protein concentration was quantified using the EZQ kit as previously described[51]; resulting data
302 were used to normalise the amount of lipid used for HPTLC analysis. Diet samples (Jazz-mix
303 and Formula 4-24) were powdered and lipids were extracted from an equal amount of each diet,
304 and dissolved in chloroform:methanol (2:1, v/v) for lipid analysis; 1.5 mg of yeast
305 (Saccharomyces cerevisiae) was added per gram of Formula 4-24 prior to lipid extraction. For
306 automated HPTLC analysis , lipid extracts were dissolved in choloroform:methanol (2:1, v/v)
307 and loaded onto silica gel 60 HPTLC plates (EMD Chemicals, Darmstadt, Germany) and
308 resolved using the CAMAG AMD 2 system. Neutral and phospholipids were resolved using
309 solvent systems that have been previously described[52]. Lipid standards (Avanta Polar Lipids,
310 Alabaster, Alabama, U.S.A.) were resolved in parallel to enable identification of resolved lipid
311 species. During the assessment of the phospholipids PE, PI, and PC, values were quantified and
312 then standardized by diving all values from each dietary stage as a percentage of the value
313 obtained for adults from the formula 4-24 diet.

314 *Data analysis*

315 To determine statistical differences between the diets, t-tests were used unless otherwise
316 noted. The unit of replication was normally the pooled group of flies tested for behavioural or
317 morphological characteristics, unless otherwise noted. Larval and pupal emergence and adult
318 survival differences between diets were tested using the non-parametric log-rank test for

survivorship (*e.g.,* Kaplan-Meir survival analysis). An alpha value of 0.05 was used for rejecting the null hypothesis of no influence of diet on characteristics of interest. Unless otherwise noted (*i.e.,* where mean values from multiple trials on the same replicate would warrant presenting a standard error of the mean), results are expressed as mean ± SD.

Effect size assessment

In order to summarize an overall effect of diet (i.e. across the numerous phenotypic measurements) with objective statistical measures and to qualify the biological significance, effect sizes were calculated using Hedge's *g*, an unbiased estimate of Cohen's *D*, obtained from the pooled standard deviations and differences in the average values for each diet [53]. Hedge's *g* was also converted into the point biserial correlation coefficient (*r*) for ease of comparison to the more familiar Pearson correlation coefficient. These effect sizes were assessed and compared using 95% confidence intervals against a null expected value of zero. An overall effect size across all experiments in this study was assessed using a technique common in meta-analyses of data sets to provide an assessment of the potential importance of diet on biological function; this was possible since the groups of flies in each of these tests were independent replicates (Field, 2001).

Results

Development and longevity

Tracking the number of third-instar larvae that emerged each day for 20 days revealed a significant difference in the average developmental time required to reach the wandering phase: 177.2 ± 2.9hours vs. 223.6 ± 1.65hours, for Jazz-mix and Formula 4-24, respectively (Fig 1, 1-way ANOVA, $F=40.17$, $P<0.0001$, Tukey post hoc $P<0.05$).). There was also a significant difference between the time required to reach the wandering stage between Formula 4-24 and control larvae (170.6 ± 2.8, $P<0.05$), but there was no difference between Jazz-mix and the control diet. There was no significant difference in the percentage of fertilized eggs that survived to wandering third-instar larvae in these experiments (Formula 4-24: 94.8 ± 4.3%, Jazz mix 91.6 ± 3.0%, control 92.4 ± 4.4%, 1-way ANOVA, $F=0.95$, $P=0.39$). Tracking the number of pupae that emerged each day for 20 days also revealed a significant difference between in the developmental time required to reach pupation: 276.1 ± 2.2 hours vs. 237.9 ± 2.0 hours, for Formula 4-24 and Jazz-mix respectively (Fig 1B, 1-way ANOVA, $F=49.24$, $P=<0.0001$, Tukey Post hoc $P<0.05$). There was also a significant difference between the time required to reach pupation between the Formula 4-24 and a control diet (231.7 ± 2.3, $P<0.05$) but not between the control and Jazz-mix diets. There was no significant difference in the percentage of fertilized eggs that survived to pupation in these experiments (Formula 4-24: 90.8 ± 5.4%, Jazz mix 89.2 ± 2.7%, control 91.6 ± 4.0%, 1-way ANOVA, $F=0.87$, $P=0.43$). Subsequently, the number of the pupae that eclosed into adults was tracked in each of these vials. There was no significant

difference between the three diets with respect to the percentage of eggs that survived to adulthood (Formula 4-24: 88.4 ±3.5%, Jazz-mix: 87.2 ± 3.7%, Control: 90.0 ± 3.9%, 1-way ANOVA, $F=1.45$, $P=0.25$). Finally, adult flies, both males and females had greater survivorship on Jazz-mix compared to the Formula 4-24 diet (Fig. 1C,D). The time to 50% death was significantly different between the two diets, for both males and females, taking on average 20 days (Formula 4-24 29.1± 7.1 days, Jazz-mix 49.7 ± 12.3 days, 1-way ANOVA, $F=37.83$, $P<0.0001$, Tukey Post Hoc $P<0.05$) longer to reach the 50% value for males, and 16 days (Formula 4-24 47.1 ± 11.1 days, 63.6 ± 8.1 days, 1-way ANOVA, $F=91.0$, $P<0.0001$, Tukey Post hoc, $P<0.05$) for females. There was also a significant difference between male and female survivorship reared on Formula 4-24 compared with a control diet (males: 50.4 ± 11.3, females: 60.0 ± 12.6, $P<0.05$).

Fecundity and virility

To assess if there were differences in the total number of eggs laid by females reared on each of the diets, an egg-laying assay was conducted. Five-day old females removed from communal vials on each of the respective diets were transferred to agar-plates for 3 days and the total number of eggs laid was assessed each day. There was no significant difference in the total number of eggs laid each day between the Formula 4-24 diet and Jazz-mix diet (234.6 ± 58.7 eggs and 238.0 ± 59.5eggs, 1-way ANOVA, $F=0.32$, $P=0.73$). There was also no significant difference between either Formula 4-24 or Jazz-mix and the control diet (241.2 ± 60.3eggs).

100 eggs from the egg-laying assay were transferred to a fresh grape-agar plate and the total number of eggs that progressed to first-instar was assessed. There was no significant difference in the percentage of eggs that progressed to first-instar larvae between any of the three

383 diets (Formula 4-24: 93.4 ± 1.3%, Jazz-mix: 95.3 ± 2.0%, 94.6 ± 1.6%,1-way ANOVA, F=3.33,
384 P=0.051).

385 A single virgin female and single virgin male were obtained from each of the diets, and
386 after 4 days they were transferred to a grape-agar plate. Subsequently, 50 eggs were transferred
387 to a fresh agar plate and the total number of eggs that progressed to first-instar larvae was
388 assessed. There was no significant difference in the percentage of eggs that progressed to first-
389 instar larvae between any of the diets (Formula 4-24: 96.6 ± 2.2, Jazz-mix: 96.9 ± 2.0, control:
390 97. 4 ± 1.0, 1-way ANOVA, F=0.42, P=0.67).

391 *Body size parameters*

392 Since there were differences in the rate of larval development between the two diets,
393 morphometric measures and weight were also assessed. Third-instar larvae reared on Jazz-mix
394 were significantly longer (Table 1, 3.688 ± 0.561 mm and 3.887 ± 0.371 mm, Formula 4-24 and
395 Jazz-mix, respectively ; Mann-Whitney, P=0.039, t_{49}=2225.5) and wider (0.661 ± 0.09 mm and
396 0.998 ± 0.10 mm, Formula 4-24 and Jazz-mix, respectively; P<0.001, T_{49}=-17.77) than those
397 reared on Formula 4-24. Larvae reared on Jazz-mix were nearly twice the weight of those fed
398 Formula 4-24 (19.415 ± 0.236 mg vs.11.171 ± 0.247 mg, N=10, t_{18}=-76.26, P<0.001), and the
399 mass of dried tissue was also significantly greater for Jazz-mix (5.506 ± 0.279 mg vs. 2.598 ±
400 0.245mg , N=10, t_{18}=-24.75, P<0.001). To test whether these differences in larval size and
401 weight were manifested in differences in adult size, the length of the 4th wing vein was
402 measured. Indeed, the size differences in larvae were found to persist into adulthood with the
403 average wing size significantly larger for flies reared on Jazz-mix than those on Formula 4-24
404 (2.025 ± 0.148 mm and 1.823 ± 0.16 mm, respectively; Mann-Whitney Rank Sum, P<0.001,

405 T=1122.0, N=40). Additionally, the weight of newly eclosed virgin females was significantly
406 different between those reared on Formula 4-24 and Jazz-mix (10 females, Formula 4-24 7.7 ±
407 0.6, Jazz-mix 9.3 ± 0.4, 1-way ANOVA, $F=39.96$, $P<0.0001$, Tukey Post Hoc $P<0.05$). A
408 significant difference was also observed between adults reared on Formula 4-24 and control diet
409 (9.0 ± 0.3, $P<0.05$); however no significant difference was observed between adults on Jazz-mix
410 and the control diet.

411 *Larval locomotory speed*

412 A modified light-avoidance assay was used to evoke escape locomotion in larvae (Fig.
413 2C). Those reared on Formula 4-24 moved significantly faster, with an average speed of 1.19 ±
414 0.21mm/s compared to those fed Jazz-mix 0.98 ± 0.15mm/s ($P=0.019$, $t_{19}=2.59$).

415 *Muscle contraction*

416 The possibility that diet alters the physiology of larval body-wall muscles, either by
417 altering the maximal force output of these muscles or by altering their responsiveness to
418 hormonal stimulation, was assessed (Fig. 2). Third-instar larvae reared on Jazz-mix generated
419 larger contractions in response to 10^{-6} M octopamine than did larvae reared on Formula 4-24 (Fig
420 2A) (6.34 ± 0.09 and 4.10 ± 0.82 mN, $P<0.001$, $t_{19}=-5.86$). In contrast, no difference was found
421 in the maximal force generated by the larval body-wall muscles in response to KCl (Fig. 2B)
422 ($P=0.6$, $t_{18}=-0.534$). Thus, larvae reared on either diet are able to generate the same total force
423 from muscle contractions but do not respond equally well to hormonal stimulation.

424 *Assays of larval behaviour*

425 Third-instar larvae reared on either Formula 4-24 or Jazz-mix were tested for thermal
426 preference (Fig 3). Within a linear thermal gradient (15-30°C; Fig 3B), larvae reared on Formula

4-24 had a significantly higher preferred temperature (17.3 ± 0.4°C) than those reared on Jazz-mix (16.0 ± 0.3°C; Fig. 3A; F= 6.587, P=0.014, N=20). Larvae reared on Formula 4-24 also travelled significantly further from a heat source (3.5 ± 0.1 cm, to 22.5°C from 38°C) than those reared on Jazz-mix (3.1 ± 0.1 cm, to 24.2°C; Fig. 4A, Kruskal-Wallis ANOVA on ranks, $P<0.01$, H=6.743, DF=1, N=54, total larvae=1080 per group). In the heat-avoidance trials, significantly fewer larvae reared on Jazz-mix passed the half-way point (2.4 cm or 30°C) of the lane (65.6 ± 3.7 vs. 79.9 ± 2.9 reared on Formula 4-24; Mann-Whitney U, $P<0.01$, T=3433.0, N=54) (Fig. 4B). In the reciprocal experiment, initiated with larvae on the cool side (22°C), larvae reared on Formula 4-24 migrated on average 0.9 ± 0.8cm or 3.1 ± 2.8°C, away from the 'cool' side (N=10 trials, 40 larvae total). It is of note that only four of 40 animals passed the half-way point. Larvae reared on Jazz-mix migrated on average 0.5 ± 0.6cm or 1.9 ± 1.9°C (N=10 trials, 40 larvae total). Here zero of the 40 animals passed the half-way point.

Assays of adult behaviour

The negative geotactic response of flies was assessed at 4 and 55 s after an abrupt drop. More adults reared on Jazz-mix climbed past the halfway point of the vial (74.0 ± 0.6) than those on Formula 4-24 (60.6 ± 0.8) 4 s after the drop (N=12, ANOVA: F statistic – 31.87, $P<0.001$) (Fig. 5A). However, 55 s after the drop, the flies performed equally well (74.7 ± 0.7 and 75.0 ± 0.8, ANOVA: F statistic – 31.87, $P>0.001$, N=72, total flies=1800) irrespective of diet (Fig. 5A).

In the thermotaxis assay, flies reared on Formula 4-24 had a preferred temperature of 21.6 ± 1.5, which was not significantly different from those fed Jazz-mix: 22.2 ± 1.6 (Fig. 6, P=0.25).

448 The CAFE assay was used in order to assess rates of food consumption between the diets.
449 Adult males reared on Jazz-mix consumed significantly more food than adult males reared on
450 Formula 4-24 (2.6 ± 0.2 vs. 2.0 ± 0.2, respectively, 1-way ANOVA, $F=56.72$, $P<0.0001$, Tukey
451 Post hoc $P<0.05$). Adult females reared on Jazz-mix also consumed significantly more food than
452 adult females reared on Formula 4-24 (3.3 ± 0.2 vs. 2.5 ± 0.2, respectively, 1-way ANOVA,
453 $F=79.00$, $P<0.0001$, Tukey Post hoc, $P<0.05$). Both male and female adults consumed
454 significantly more food when reared on the control diet compared to Formula 4-24 ($P<0.05$),
455 however, there was no significant difference in the amount of food consumed between males or
456 females reared on Jazz-mix median and those reared on a control diet.

457 As a final behavioural assay, adult olfactory learning and memory was assessed using a
458 modified version of the Tully and Quinn T-maze[35]. Adults reared on either diet performed
459 equally well even when tested 10 min after training (Figure 7; 2 minutes: Formula 4-24 0.7 ± 0.0
460 Jazz-mix 0.70 ± 0.0; ANOVA: $P=0.699$, $F=0.601$, $N=10$, total flies>1000). We also assessed
461 learning and memory 5 and 10 minutes after training (5 minutes Formula 4-24 0.7 ± 0.0, Jazz-
462 mix 0.7 ± 0.0; 10 minutes Formula 4-24 0.6 ± 0.0, Jazz-mix 0.7 ± 0.0).

463 *Diet composition*

464 Both diets were initially chemically assayed for total moisture, protein, ash, fat and
465 carbohydrate content (Table 2). There were apparent differences in all properties examined. The
466 Formula 4-24 diet contained proportionally more protein, ash, fat and carbohydrates than did
467 Jazz-mix, whereas Jazz-mix contained proportionally more moisture. Next, the raw starting
468 material for each diet was assessed using HPTLC for to test for differences in: cholesterol,
469 monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) phosphatidylcholine

(PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE; Table 3). Significant differences were observed for all seven components that were assessed. Cholesterol, and MAG were significantly higher in the Jazz-mix diet, however, TAG, DAG, PE, PI, and PC were all significantly higher in the Formula 4-24 diet.

Tissue from larval, pupae, and adults were then compared to determine if the differences in starting material manifested itself as a significant difference in the *Drosophila* tissue (Table 4). There was no significant difference in cholesterol concentrations between the two diets during any of the developmental stages assessed (Table 4). We also assessed the tissue for differences in other critical membrane lipids (Table 4). There were no significant differences in either MAG or TAG levels during any of the developmental stages assessed. DAG levels were higher in larvae reared on Formula 4-24 which correlates with higher DAG in their diet. However, DAG levels were comparable in pupae reared on both diets, but higher in adults reared on Jazz-mix compared to Formula 4-24. Both PE and PC were significantly higher in tissue isolated from animals reared on the Formula 4-24 diet during all three life stages assessed, consistent with the differences in dietary composition. There was however no differences in PI concentrations in tissue from any of the life stages assessed.

Overall Diet Effect: Effect size estimates

Diet influenced multiple phenotypes (summarised in Table 5). The overall *z* score from a meta-analysis of effect size was 15.76, supporting a strong overall diet effect. Indeed, in 11 out of the 14 primary experimental tests, the biological effect of diet (Hedge's *g*) was significantly different from zero.

Discussion:

Here we have tested the hypothesis that rearing *D. melanogaster* on commercial media of differing chemical composition can have profound effects on a multitude of phenotypic characteristics. Our data demonstrate that these two media yielded significant differences in development, morphology, physiology, and behaviour. In almost 80% of the phenotypic comparisons we observed significant biological effects (Table 5). Notably, while learning and memory were not affected in adult flies, morphological parameters as well as developmental time as larvae and longevity as adults were significantly different, favouring flies reared on Jazz-mix as opposed to Formula 4-24.

We have demonstrated that diet can have a profound effect on a number of metrics for both the larval and adult forms of *Drosophila melanogaster*. Some of the more important characteristics in terms of fitness and survivorship are speed of development, fecundity and longevity. Other researchers have demonstrated an inverse relationship between longevity and reproduction rate for *Drosophila*, with lower nutritional diets favouring longevity at the expense of fecundity[8,11,54]. Based on such findings, we might expect the longer lifespan in flies raised on Jazz-mix to reflect dietary restriction compared to Formula 4-24, and, indeed, Jazz-mix had lower levels of protein, carbohydrate and fat than did Formula 4-24 (Table 2). On the other hand, nutrient availability, rather than total protein, carbohydrate or lipid levels, must also be considered. Increased nutrient availability triggers a rapid elevation in reproductive activity in

515 *Drosophila*, increasing both re-mating frequency and the number of eggs laid by females[8,11].
516 Both our larval and pupal emergence data indicate that Jazz-mix larvae emerge faster, but there
517 was no significant difference between the number of larvae or pupae produced on the two diets
518 after about 30 days (Fig 1). The total number of eggs laid, as well as the percentage of eggs that
519 progressed from eggs to first-instars did not differ between the two diets. Thus, fecundity does
520 not appear to be altered as a consequence of these diets. It is noteworthy that larvae on Jazz-mix
521 are nearly twice the mass of those on Formula 4-24, and they do not require more time to achieve
522 this size. It is therefore likely that Jazz-mix provides greater nutrient availability, potentially
523 attributable to differences in media preparation; larvae would thus spend less time converting the
524 substrates into metabolized nutrient stores than when raised on Formula 4-24. Previous work has
525 also demonstrated that nutrient-poor diets can result in smaller sized adults[55]; the lower larval
526 mass and smaller adult size of *Drosophila* reared on Formula 4-24 may thus represent poorer
527 nutrition. It is also noteworthy that as adults the Jazz-mix flies consume food at a significantly
528 greater rate. This added food intake may directly contribute to their improved health or it may
529 simply be a product of their significantly greater size, as observed in previous reports[56]. Taken
530 together, our findings indicate that in spite of lower fat, carbohydrate and protein concentrations,
531 the Jazz-mix food contains a nutrient balance that optimises reproduction rates without
532 negatively influencing lifespan, or conversely that Formula 4-24 hinders reproduction rates and
533 lifespan due to malnutrition rather than dietary restriction[54].

534 To elucidate whether rearing flies on the two diets resulted in physiological differences in
535 muscle function, we examined contractions of larval body-wall muscles in response to
536 exogenous application of a well-known hormone and neuromodulator, octopamine (Fig. 2).
537 Octopamine has been implicated as the 'fight-or-flight' hormone in *Drosophila*, and induces a

very large response in larval body-wall muscle[45,57]. Additionally, octopamine has been implicated in: coordinating thermoprotection in insects[58], associative olfactory memory in *Drosophila*[59], locomotion[60], and other physiologically and behaviourally relevant parameters[61–64]. Application of 10^{-6} M octopamine (previously demonstrated to be the EC_{50} for inducing contractions in *Drosophila* body wall muscles[45]) induced a significantly greater change in tonus in larvae reared on Jazz-mix than in those reared on Formula 4-24. In contrast, rearing flies in the different media did not alter the maximal force of contraction induced by direct depolarization with KCl, indicating that diet did not alter the overall capacity of the muscles for force generation. These results suggest that diet may influence the responsiveness of muscles to internal signalling molecules and, potentially, that diet can affect relevant cellular signalling pathways. Diet is known to influence hormonal signalling in *Drosophila*. One critical dietary requirement is cholesterol, required for synthesis of numerous hormonal and signalling molecules, such as the production of ecdysone and 20-hydroxyecdysone associated with molting and pupation[65]. Interestingly, Formula 4-24 has previously been used as a low-cholesterol diet, and is reported to contain low levels of other plant sterols[66]. Consequently, cholesterol and other plant derived sterols are an area of dietary interest, particularly in insects, which require a dietary source for development and survival[66,67]. Plant sterols have also been of interest in human research as potential treatments for hypercholesterolemia[68].

As muscle responsiveness to exogenous application of octopamine was altered as a consequence of different dietary media, we postulated that overall muscle performance, i.e. locomotion or crawling rate, might be affected by differences in diet (Fig 2). However, even though third-instar body wall muscles produced stronger contractions in response to octopamine in those larvae reared on Jazz-mix, those reared on Formula 4-24 had faster crawling rates.

561 Nevertheless, these velocity values are comparable to what has previously been reported
562 (~1mm/s[69]). Thus, differences in crawling rates may be attributable to the dramatic size
563 difference between larvae reared on the two diets, with Jazz-mix larvae being nearly twice the
564 weight. It is also of note that because light-avoidance (Fig 2) is mediated by a cellular signalling
565 pathway, the differences we observed in mobility may not only be influenced by mass but also
566 by differences in intra- and inter-cellular signalling[70].

567 Although the thermal preference values for third-instar larvae measured here for both
568 diets were similar to previous determinations, we observed that larvae reared on Jazz-mix had a
569 slightly lower, yet significantly different preferred temperature value relative to those maintained
570 on Formula 4-24 (Figs 3)[28,29]. Subsequent to the larval preferred temperature assay, we subjected
571 larvae to a thermal retreat assay and found that animals reared on Formula 4-24 travelled
572 significantly further away from a high temperature extreme. Thus, despite a significantly lower
573 preferred temperature value for larvae reared on Jazz-mix, these did not exhibit greater thermal
574 avoidance behaviour, although there was a statistical difference in crawling rates which might be
575 interpreted as a potential confounding influence of the thermal avoidance assay. However, the
576 length of time larvae were subjected to the thermal avoidance assay (2 min) was more than
577 sufficient to mitigate these slight alterations in locomotory capacity, and thus this result is more
578 likely indicative of a greater tolerance or reduced detection of warmer temperatures in Jazz-mix
579 larvae.

580 As adults, differential rearing media does not appear to alter thermal preference as both
581 groups preferred a value of ~22°C, which is similar to previous research[32]. We did not dissociate
582 between males and females, however, the preferred temperature value established here has
583 previously been demonstrated to be similar for males and females of *D. melanogaster*[32,33]. Our

adult geotaxis results revealed a significant reduction in the fast (4s) climbing ability of adults reared on the Formula 4-24 diet, however, this effect was not long lasting (i.e. after 55 s the distribution of flies in the tube was identical between both groups). Although we did not examine muscle in adults, third instar larvae raised on Formula 4-24 showed no signs of muscle damage, as maximal force generation was not impaired, and crawling speed was greater in this group than in the Jazz-mix group. Interestingly, a progressive diminishment in negative geotactic behaviour is known to occur with age in adult *Drosophila*, even during time periods of minimal mortality[34]. Since our data do not indicate any diet-induced differences in the capacity for learning and memory, the reduction in initial climbing speed in adults from the Formula 4-24 may be related to the accelerated mortality rate observed in the Formula 4-24 reared flies. Lastly, because adults reared on Formula 4-24 are significantly smaller than those reared on Jazz-mix, the impaired geotactic response cannot be attributed to greater body mass. Taken together, our results indicate that diet can influence the behaviour of adult *Drosophila*, and these differences are at least initially attributable to differences in morphology or development of the animal.

The data on diet composition represent an initial effort to identify dietary factors that influence development and physiological functions, using the *Drosophila* model system. Notably, Jazz-mix contained proportionally greater moisture than Formula 4-24; larvae prefer moist over dry substrates[71], and moisture can affect the number of pupae and rate of mortality[72] along with many other factors[73–75]. Interestingly, all other macro-nutritional components were proportionally greater in the Formula 4-24 diet, at least in terms of traditional chemical analyses (Table 2). Of the four remaining nutritional components, fat content is probably the most extensively investigated. Birse et al. (2010)[76] demonstrated that *Drosophila* fed with a high-fat diet exhibit increased triglycerides and altered insulin/glucose homeostasis that resulted in

607 cardiac lipid accumulation, reduced cardiac contractility, conduction blocks, and structural
608 pathologies. Increasing fat content has also been shown to decrease lifespan[15], and alter stress
609 responses[77] in *Drosophila*. Consequently, we decided to focus on some of the critical
610 components within the fat profile, and examine how they differ in both the raw material as well
611 as how these changes translate to the animal. In terms of composition, cholesterol and MAG
612 were significantly higher in the Jazz-mix diet, while TAG, DAG, PE, PI, and PC were
613 significantly higher in the Formula 4-24 diet. However, only a few of these molecules were
614 significantly different when we assessed their concentrations in whole bodies at each of the three
615 developmental stages assessed.

616 Of the seven phospholipid, glyceride, or sterol molecules investigated, three differed
617 significantly in the different developmental stages. Both PE and PC were significantly more
618 abundant in the larvae and pupal stages of development in animals reared on Formula 4-24. This
619 is anticipated as both these molecules were in greater abundance in the Formula 4-24 diet. PC
620 and PE are both very abundant in biological membranes, with PC being the principle
621 phospholipid in animals and PE comprising roughly 25% of the phospholipids in membranes.
622 They are both synthesized via the CDP-ethanolamine pathway, using ethanolamine as their
623 substrate[78]. They serve a variety of biological and biophysical roles for cells, including
624 regulating membrane fluidity, permeability and fusion. While cholesterol was not significantly
625 different during any of the developmental stages, there was a large difference in the larval tissue.
626 This lack of difference might be attributable to an inability of the technique utilized to
627 differentiate between closely related sterols, including ergosterol.

628 Perhaps the most notable change in phospholipid composition was a significant change in
629 DAG. There was approximately twice the amount of DAG in the formula 4-24 diet compared to

630 Jazz-mix, which directly correlated to a doubling of DAG in the larval tissue from animals reared
631 on the Formula 4-24 diet. Conversely, there was no significant difference in DAG during the
632 pupal stage, but nearly three-times the amount of DAG in adult animals reared on Jazz-mix
633 compared to those reared on Formula 4-24. DAG is a heavily investigated molecule that is
634 implicated in a number of vital cellular pathways, perhaps most notably DAG synthesized via the
635 hydrolysis of phospholipid phosphatidylinositol 4,5-biphosphate (PIP2) by PLC producing IP_3
636 and DAG. Subsequently DAG actives PKC leading to a number of downstream effects including
637 lipid storage, fatty acid oxidation, altered muscle activity through interactions with troponin,
638 SERCA, and myosin, or it can act as a transcription factor for a number of genes[79]. DAG levels
639 have been associated with lifespan by altering rapamycin (TOR) levels, one of the downstream
640 targets of PKC. Lin et al, (2014) demonstrated in both *Drosophila* and *C. Elegans*, that
641 overexpressing diacylglycerol lipase (DAGL) or reducing the expression of diacylglycerol kinase
642 (DGK) extends lifespan[80]. DAG levels are also associated with phototransduction through
643 interaction with rolling blackout, an integral membrane lipase, or other transient receptor
644 potential channels (TRP/TRPL[80,81]). Such a combination of effects may thus explain why the
645 flies reared on the Jazz-mix diet have a longer lifespan. Given that cholesterol, TAG, MAG and
646 PI levels did not differ across all three stages of development, irrespective of diet, emphasizes a
647 critical role for regulating the amounts of these lipids within the animal. This is even more
648 striking given that the starting media upon which these animals were reared contained
649 significantly different amounts of these lipidic components, with MAG and cholesterol being
650 more abundant in Jazz-mix, and DAG and PI being more abundant in formula 4-24.

651 Overall, our observations confirm that two standard diets can significantly alter
652 fundamental processes associated with development, morphology, physiology and behaviour in

D. melanogaster. The observed effects are more prevalent in larvae, but persist into adulthood and were especially clear with respect to development and fly lifespan. Assessments of the dietary components that may underlie these developmental, morphological, and behavioural differences revealed a number of putative factors - PC, PI and DAG - that differ significantly in the tissue of these animals. Nonetheless, we report that altering dietary components in food media can result in a multitude of confounding implications and leave it to the wider scientific community to consider how inter-lab differences in dietary media may influence results across a broad range of study types.

Acknowledgements:

This work was supported by a Queen Elizabeth II scholarship in science and technology and National Science and Engineering Research Council of Canada (NSERC) PGS funding to KGO, and a NSERC undergraduate student research award to OKL. JRC acknowledges initial support from the Canadian Institutes of Health Research, and the WSU School of Medicine. PSA was supported by a PhD Scholarship from the WSU School of Medicine. NSERC grants to AJM (46292), and GJT (262 087). GJT would also like to acknowledge Brock University Chancellor's Chair funding.

References:

1. Kopec, S. On the Influence of Intermittent Starvation on the Longevity of the Imaginal Stage of Drosophila Melanogaster. *J. Exp. Biol.* **5,** 204–211 (1928).

2. Baumberger, J. P. & Glaser, R. W. THE REARING OF DROSOPHILA AMPELOPHILA LOEW ON SOLID MEDIA. *Science* **45,** 21–2 (1917).

3. Doubal, S. & Klemera, P. The effect of antioxidants and dietary restriction on mortality curves. *Age (Omaha).* **22,** 101–5 (1999).

4. Anagnostou, C., Dorsch, M. & Rohlfs, M. Influence of dietary yeasts on Drosophila melanogaster life-history traits. *Entomol. Exp. Appl.* **136,** 1–11 (2010).

5. Hartman, T. R., Strochlic, T. I., Ji, Y., Zinshteyn, D. & O'Reilly, A. M. Diet controls Drosophila follicle stem cell proliferation via Hedgehog sequestration and release. *J. Cell Biol.* **201,** 741–57 (2013).

6. Carvalho, M. *et al.* Survival strategies of a sterol auxotroph. *Development* **137,** 3675–85 (2010).

7. Powsner, L. The Effects of Temperature on the Durations of the Developmental Stages of Drosophila melanogaster on JSTOR. *Physiological Zoology* 474–520 (1935).

8. Chippindale, A. K., Leroi, A. M., Kim, S. B. & Rose, M. R. Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. *J. Evol. Biol.* **6,** 171–193 (1993).

9. Fanson, B. G., Weldon, C. W., Pérez-Staples, D., Simpson, S. J. & Taylor, P. W. Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (Bactrocera tryoni). *Aging Cell* **8,** 514–23 (2009).

10. Vigne, P. & Frelin, C. Diet dependent longevity and hypoxic tolerance of adult Drosophila melanogaster. *Mech. Ageing Dev.* **128,** 401–6

11. Chapman, T. & Partridge, L. Female fitness in Drosophila melanogaster: an interaction between the effect of nutrition and of encounter rate with males. *Proc. Biol. Sci.* **263,** 755–9 (1996).

12. Chhabra, R., Kolli, S. & Bauer, J. H. Organically grown food provides health benefits to Drosophila melanogaster. *PLoS One* **8,** e52988 (2013).

13. Riddiford, L. M. & Ashburner, M. Effects of juvenile hormone mimics on larval development and metamorphosis of Drosophila melanogaster. *Gen. Comp. Endocrinol.* **82,** 172–83 (1991).

14. Alpatov, W, W. Experimental Studies on the Duration of Life. XIII. The Influence of Different Feeding during the Larval and Imaginal Stages on the Duration of Life of the Imago of Drosophila melanogaster on JSTOR. *The American Naturalist* 37–55 (1930).

15. Driver, C. J. & Cosopodiotis, G. The effect of dietary fat on longevity of Drosophila melanogaster. *Exp. Gerontol.* **14,** 95–100 (1979).

16. Testa, N. D., Ghosh, S. M. & Shingleton, A. W. Sex-specific weight loss mediates sexual size dimorphism in Drosophila melanogaster. *PLoS One* **8,** e58936 (2013).

17. Cortese, M. D., Norry, F. M., Piccinali, R. & Hasson, E. Direct and correlated responses to artificial selection on developmental time and wing length in Drosophila buzzatii. *Evolution* **56,** 2541–7 (2002).

18. Kalmus, H. The Resistance to Desiccation of Drosophila Mutants Affecting Body Colour. *Proc. R. Soc. B Biol. Sci.* **130,** 185–201 (1941).

19. Suster, M. L. & Bate, M. Embryonic assembly of a central pattern generator without sensory input. *Nature* **416,** 174–8 (2002).

20. Heimbeck, G., Bugnon, V., Gendre, N., Häberlin, C. & Stocker, R. F. Smell and taste perception in Drosophila melanogaster larva: toxin expression studies in chemosensory neurons. *J. Neurosci.* **19,** 6599–609 (1999).

21. Shaver, S. A., Varnam, C. J., Hilliker, A. J. & Sokolowski, M. B. The foraging gene affects adult but not larval olfactory-related behavior in Drosophila melanogaster. *Behav. Brain Res.* **95,** 23–9 (1998).

22. Gerber, B. *et al.* Visual learning in individually assayed Drosophila larvae. *J. Exp. Biol.* **207,** 179–88 (2004).

23. Pereira, H. S. & Sokolowski, M. B. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5044–6 (1993).

24. Heiman, R. G. *et al.* Spontaneous avoidance behavior in Drosophila null for calmodulin expression. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2420–5 (1996).

25. Suster, M. L., Martin, J.-R., Sung, C. & Robinow, S. Targeted expression of tetanus toxin reveals sets of neurons involved in larval locomotion in Drosophila. *J. Neurobiol.* **55,** 233–46 (2003).

26. Caldwell, J. C., Miller, M. M., Wing, S., Soll, D. R. & Eberl, D. F. Dynamic analysis of larval locomotion in Drosophila chordotonal organ mutants. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 16053–8 (2003).

27. Carhan, A., Reeve, S., Dee, C. T., Baines, R. A. & Moffat, K. G. Mutation in slowmo causes defects in Drosophila larval locomotor behaviour. *Invert. Neurosci.* **5,** 65–75 (2004).

28. Liu, L., Yermolaieva, O., Johnson, W. A., Abboud, F. M. & Welsh, M. J. Identification and function of thermosensory neurons in Drosophila larvae. *Nat. Neurosci.* **6,** 267–73 (2003).

29. Kwon, Y., Shim, H.-S., Wang, X. & Montell, C. Control of thermotactic behavior via coupling of a TRP channel to a phospholipase C signaling cascade. *Nat. Neurosci.* **11,** 871–3 (2008).

30. Lilly, M. & Carlson, J. smellblind: a gene required for Drosophila olfaction. *Genetics* **124,** 293–302 (1990).

31. Gong, Z. *et al.* A molecular diffusion based utility model for Drosophila larval phototaxis. *Theor. Biol. Med. Model.* **9,** 3 (2012).

32. Sayeed, O. & Benzer, S. Behavioral genetics of thermosensation and hygrosensation in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6079–84 (1996).

33. Yamamoto, A. & Ohba, S. Strategic differences in thermal adaptation between two Drosophila species, D. virilis and D. immigrans. *Oecologia* **52,** 333–339 (1982).

34. Skandalis, D. A., Stuart, J. A. & Tattersall, G. J. Responses of Drosophila melanogaster to atypical oxygen atmospheres. *J. Insect Physiol.* **57,** 444–51 (2011).

35. Tully, T. & Quinn, W. G. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J. Comp. Physiol. A.* **157,** 263–77 (1985).

36. Sokolowski, M. B. Social interactions in "simple" model systems. *Neuron* **65,** 780–94 (2010).

37. Chen, S., Lee, A. Y., Bowens, N. M., Huber, R. & Kravitz, E. A. Fighting fruit flies: A model system for the study of aggression. *Proc. Natl. Acad. Sci.* **99,** 5664–5668 (2002).

38. Greenspan, R. J. & Ferveur, J. F. Courtship in Drosophila. *Annu. Rev. Genet.* **34,** 205–232 (2000).

39. Cosens, D. Blue adaptation: an experimental tool for the study of visual receptor mechanisms and behaviour of Drosophila. *Biophys. Struct. Mech.* **5,** 211–22 (1979).

40. Ritchie, Halsey & Gleason. Drosophila song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in D. melanogaster song. *Anim. Behav.* **58,** 649–657 (1999).

41. Keene, A. C. & Waddell, S. Drosophila olfactory memory: single genes to complex neural circuits. *Nat. Rev. Neurosci.* **8,** 341–354 (2007).

42. Montell, C. A taste of the Drosophila gustatory receptors. *Curr. Opin. Neurobiol.* **19,** 345–53 (2009).

43. Nichols, C. D., Becnel, J. & Pandey, U. B. Methods to assay Drosophila behavior. *J. Vis. Exp.* (2012). doi:10.3791/3795

44. Monastirioti, M. *et al.* Octopamine immunoreactivity in the fruit fly Drosophila melanogaster. *J. Comp. Neurol.* **356,** 275–87 (1995).

45. Ormerod, K. G., Hadden, J. K., Deady, L. D., Mercier, A. J. & Krans, J. L. Action of

octopamine and tyramine on muscles of Drosophila melanogaster larvae. *J. Neurophysiol.* **110,** (2013).

46. Clark, J., Milakovic, M., Cull, A., Klose, M. K. & Mercier, A. J. Evidence for postsynaptic modulation of muscle contraction by a Drosophila neuropeptide. *Peptides* **29,** 1140–9 (2008).

47. Jan, L. Y. & Jan, Y. N. Properties of the larval neuromuscular junction in Drosophila melanogaster. *J. Physiol.* **262,** 189–214 (1976).

48. Hong, S.-T. *et al.* Histamine and its receptors modulate temperature-preference behaviors in Drosophila. *J. Neurosci.* **26,** 7245–56 (2006).

49. Bligh, E. G. & Dyer, W. J. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Can. J. Biochem. Physiol.* **37,** 911–917 (1959).

50. Churchward, M. A., Rogasevskaia, T., Höfgen, J., Bau, J. & Coorssen, J. R. Cholesterol facilitates the native mechanism of Ca2+-triggered membrane fusion. *J. Cell Sci.* **118,** 4833–48 (2005).

51. Wright, E. P. *et al.* Top-down proteomics: enhancing 2D gel electrophoresis from tissue processing to high-sensitivity protein detection. *Proteomics* **14,** 872–89 (2014).

52. Churchward, M. A. *et al.* Specific lipids supply critical negative spontaneous curvature--an essential component of native Ca2+-triggered membrane fusion. *Biophys. J.* **94,** 3976–86 (2008).

53. Fritz, C. O., Morris, P. E. & Richler, J. J. Effect size estimates: current use, calculations, and interpretation. *J. Exp. Psychol. Gen.* **141,** 2–18 (2012).

54. Piper, M. D. W., Skorupa, D. & Partridge, L. Diet, metabolism and lifespan in Drosophila. *Exp. Gerontol.* **40,** 857–62 (2005).

55. Vijendravarma, R. K., Narasimha, S. & Kawecki, T. J. Effects of parental larval diet on egg size and offspring traits in Drosophila. *Biol. Lett.* **6,** 238–41 (2010).

56. Ja, W. W. *et al.* Prandiology of Drosophila and the CAFE assay. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 8253–6 (2007).

57. Roeder, T. Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* **50,** 447–77 (2005).

58. Armstrong, G. A. B. & Meldrum Robertson, R. A role for octopamine in coordinating thermoprotection of an insect nervous system. *J. Therm. Biol.* **31,** 149–158 (2006).

59. Honda, T., Lee, C.-Y., Yoshida-Kasikawa, M., Honjo, K. & Furukubo-Tokunaga, K. Induction of associative olfactory memory by targeted activation of single olfactory neurons in Drosophila larvae. *Sci. Rep.* **4,** 4798 (2014).

60. Saraswati, S., Fox, L. E., Soll, D. R. & Wu, C.-F. Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae. *J. Neurobiol.* **58,** 425–41 (2004).

834 61. Blumenthal, E. M. Regulation of chloride permeability by endogenously produced
835 tyramine in the Drosophila Malpighian tubule. *Am. J. Physiol. Cell Physiol.* **284,** C718-28
836 (2003).

837 62. Wierenga, J. M. & Hollingworth, R. M. Octopamine uptake and metabolism in the insect
838 nervous system. *J. Neurochem.* **54,** 479–89 (1990).

839 63. Nagaya, Y., Kutsukake, M., Chigusa, S. I. & Komatsu, A. A trace amine, tyramine,
840 functions as a neuromodulator in Drosophila melanogaster. *Neurosci. Lett.* **329,** 324–8
841 (2002).

842 64. Certel, S. J. *et al.* Octopamine neuromodulatory effects on a social behavior decision-
843 making network in Drosophila males. *PLoS One* **5,** e13248 (2010).

844 65. Garen, A., Kauvar, L. & Lepesant, J.-A. Roles of ecdysone in Drosophila development
845 (steroid hormone/temperature-sensitive mutants). *PNAS* **74,** 5099–5103 (1977).

846 66. Horner, M. A. *et al.* The Drosophila DHR96 nuclear receptor binds cholesterol and
847 regulates cholesterol homeostasis. *Genes Dev.* **23,** 2711–6 (2009).

848 67. Bouvaine, S. *et al.* The physiology of sterol nutrition in the pea aphid Acyrthosiphon
849 pisum. *J. Insect Physiol.* **58,** 1383–9 (2012).

850 68. Genser, B. *et al.* Plant sterols and cardiovascular disease: a systematic review and meta-
851 analysis. *Eur. Heart J.* **33,** 444–51 (2012).

852 69. Cheng, L. E., Song, W., Looger, L. L., Jan, L. Y. & Jan, Y. N. The role of the TRP
853 channel NompC in Drosophila larval and adult locomotion. *Neuron* **67,** 373–80 (2010).

854 70. Keene, A. C. *et al.* Distinct visual pathways mediate Drosophila larval light avoidance and
855 circadian clock entrainment. *J. Neurosci.* **31,** 6527–34 (2011).

856 71. Graf, S. A. & Sokolowski, M. B. Rover/sitterDrosophila melanogaster larval foraging
857 polymorphism as a function of larval development, food-patch quality, and starvation. *J.*
858 *Insect Behav.* **2,** 301–313 (1989).

859 72. Sameoto, D. D. & Miller, R. S. Selection of Pupation Site by Drosophila Melanogaster
860 and D. Simulans. *Ecology* **49,** 177–180 (1968).

861 73. Liu, L. *et al.* Drosophila hygrosensation requires the TRP channels water witch and
862 nanchung. *Nature* **450,** 294–8 (2007).

863 74. Pandey, M. B. & Singh, B. N. Effect of biotic and abiotic factors on pupation height in
864 four species of Drosophila. *Indian J. Exp. Biol.* **31,** 912–7 (1993).

865 75. Spassky, B. Effect of Temperature and Moisture Content of the Nutrient Medium on the
866 Viability of Chromosomal Types in Drosophila pseudoobscura on JSTOR. *The American*
867 *Naturalist* 177–180 (1951).

868 76. Birse, R. T. *et al.* High-fat-diet-induced obesity and heart dysfunction are regulated by the
869 TOR pathway in Drosophila. *Cell Metab.* **12,** 533–44 (2010).

77. Heinrichsen, E. T. & Haddad, G. G. Role of high-fat diet in stress response of Drosophila. *PLoS One* **7,** e42587 (2012).

78. Bleijerveld, O. B., Brouwers, J. F. H. M., Vaandrager, A. B., Helms, J. B. & Houweling, M. The CDP-ethanolamine pathway and phosphatidylserine decarboxylation generate different phosphatidylethanolamine molecular species. *J. Biol. Chem.* **282,** 28362–72 (2007).

79. McLaughlin, S., Wang, J., Gambhir, A. & Murray, D. PIP(2) and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* **31,** 151–75 (2002).

80. Lin, Y.-H. *et al.* Diacylglycerol lipase regulates lifespan and oxidative stress response by inversely modulating TOR signaling in Drosophila and C. elegans. *Aging Cell* **13,** 755–64 (2014).

81. Leung, H.-T. *et al.* DAG lipase activity is necessary for TRP channel regulation in Drosophila photoreceptors. *Neuron* **58,** 884–96 (2008).

Table 1: Morphological features of *Drosophila* reared on two commercially available diets.

Feature	Formula 4-24	Jazz-mix	P-value
Larval wet mass (mg)	11.17 ± 0.25	19.42 ± 0.24	P<0.001
Larval dry mass (mg)	2.60 ± 0.25	5.51 ± 0.28	P<0.001
Larval length (mm)	3.69 ± 0.56	3.89 ± 0.37	N/S
Larval width (mm)	0.66 ± 0.09	1.00 ± 0.10	P<0.001
Wing measurement (mm) (4^{th} vein – reference)	1.82 ± 0.16	2.03 ± 0.15	P<0.001
Wing length males (mm)	1.77 ± 0.04	1.90 ± 0.06	P<0.001
Wing length females (mm)	1.88 ± 0.04	2.16 ± 0.08	P<0.001
Adult wet mass (mg)	7.75 ± 0.57	9.3 ± 0.35	P<0.001

Table 2: General composition of Formula 4-24 and Jazz-mix.

Breakdown	Formula 4-24	Jazz-mix	Ratio of F4-24:Jazz
Moisture	75.73	81.69	0.9
Protein %	1.91	0.80	2.4
Ash %	1.05	0.49	2.1
Fat %	0.12	0.01	12
Carbohydrates % (calc)	21.19	17.01	1.2

Table 3: Major lipidic species in Formula 4-24 and Jazz-mix. Values expressed as arbitrary units. *-P<0.05, **P<0.01, ***P<0.001

	Formula 4-24	Jazz-mix	Ratio F424:Jazz
Cholesterol (AU)	1 747 204 ± 37 086	4 382 774 ± 36 759	0.4***
TAG (AU)	2 813 326 ± 27 282	2 291 746 ± 85 901	1.2***
DAG (AU)	1 263 798 ± 15 115	722 640 ± 9 970	1.7***
MAG (AU)	1 434 098 ± 58 165	2 512 646 ± 43 899	0.6**
PE	1 269 962 ± 22983	159 647 ± 9979	8.0***
PI	1 151 838 ± 26 744	310 915 ± 38 668	3.7***
PC	1 126 151 ± 44 230	325 646 ± 34 991	3.5***

Table 4: Body composition of animals reared on Formula 4-24 and Jazz-mix (Units: Cholesterol, TAG, DAG, MAG - ug/mg protein; PE, PI, PC – % of Adult formula 4-24 AU). *-P<0.05, **P<0.01, ***P<0.001

	Formula 4-24	Jazz-mix	Ratio
Larvae			
Cholesterol	4.6 ± 0.3	6.1 ± 0.5	0.8
TAG	90.7 ± 2.7	80.8 ± 8.3	1.1
DAG	1.6 ± 0.1	0.8 ± 0.2	2.0 *
MAG	85.0 ± 6.5	66.9 ± 12.2	1.2
PE	108.5 ± 55.5	44.6 ± 35.9	2.4 *
PI	230.8 ± 135.5	126.7 ± 128.1	1.8
PC	113.6 ± 49.4	59.8 ± 46.5	1.9 *
Pupae			
Cholesterol	2.3 ± 0.5	2.9 ± 0.4	0.8
TAG	71.1 ± 10.3	63.2 ± 1.7	1.1
DAG	0.6 ± 0.1	0.8 ± 0.2	0.8
MAG	53.0 ± 7.1	57.6 ± 19.24	0.9
PE	46.1 ± 28.0	11.5 ± 7.8	4.0 *
PI	92.7 ± 55.0	24.2 ± 20	3.8
PC	48.8 ± 28.2	15.8 ± 11.5	3.1 *
Adults			
Cholesterol	3.5 ± 0.4	3.1 ± 0.5	1.1
TAG	61.0 ± 12.4	68.4 ± 16.0	0.9
DAG	0.4 ± 0.0	1.3 ± 0.1	0.3 *
MAG	70.3 ± 16.5	49.9 ± 14.5	1.4
PE	44.6 ± 36.0	49.7 ± 10.5	0.9
PI	100.0 ± 91.7	54.1 ± 12.9	1.8
PC	100.0 ± 76.6	63.7 ± 14.7	1.6 *

965 **Table 5. Effect sizes of the influence of diet on developmental, behavioural, and**
966 **physiological parameters**

Comparison	Phenotype	Hedge's g	r	Significant
Larval Wet Mass	Morphology	32.66	0.9982849	Yes
Larval Dry Mass	Morphology	10.60	0.9840578	Yes
Larval Width	Morphology	3.53	0.8714722	Yes
Adult Wing Length	Morphology	1.30	0.5484725	Yes
Adult Wet Mass	Morphology	3.12	0.8519213	Yes
Egg Laying	Development	0.05	0.027921	No
Larva Emergence	Development	3.28	0.86	Yes
Pupa Emergence	Development	4.39	0.92	Yes
Adult survivorship	Lifespan	1.95	0.71	Yes
Larva Thermal Preference	Sensory	0.80	0.36	Yes
Adult Thermal Preference	Sensory	0.38	0.18	No
Larval Thermal Retreat	Neuromuscular	0.73	0.34	Yes
Adult Geotaxis	Neuromuscular	2.49	0.77	Yes
Muscle Basal Tonus	Neuromuscular	2.51	0.77	Yes
Muscle Maximum	Neuromuscular	0.23	0.11	No
Feeding Rate	Feeding	1.87	0.6899348	Yes
Learning Index	Neurological	0.34	0.16	No

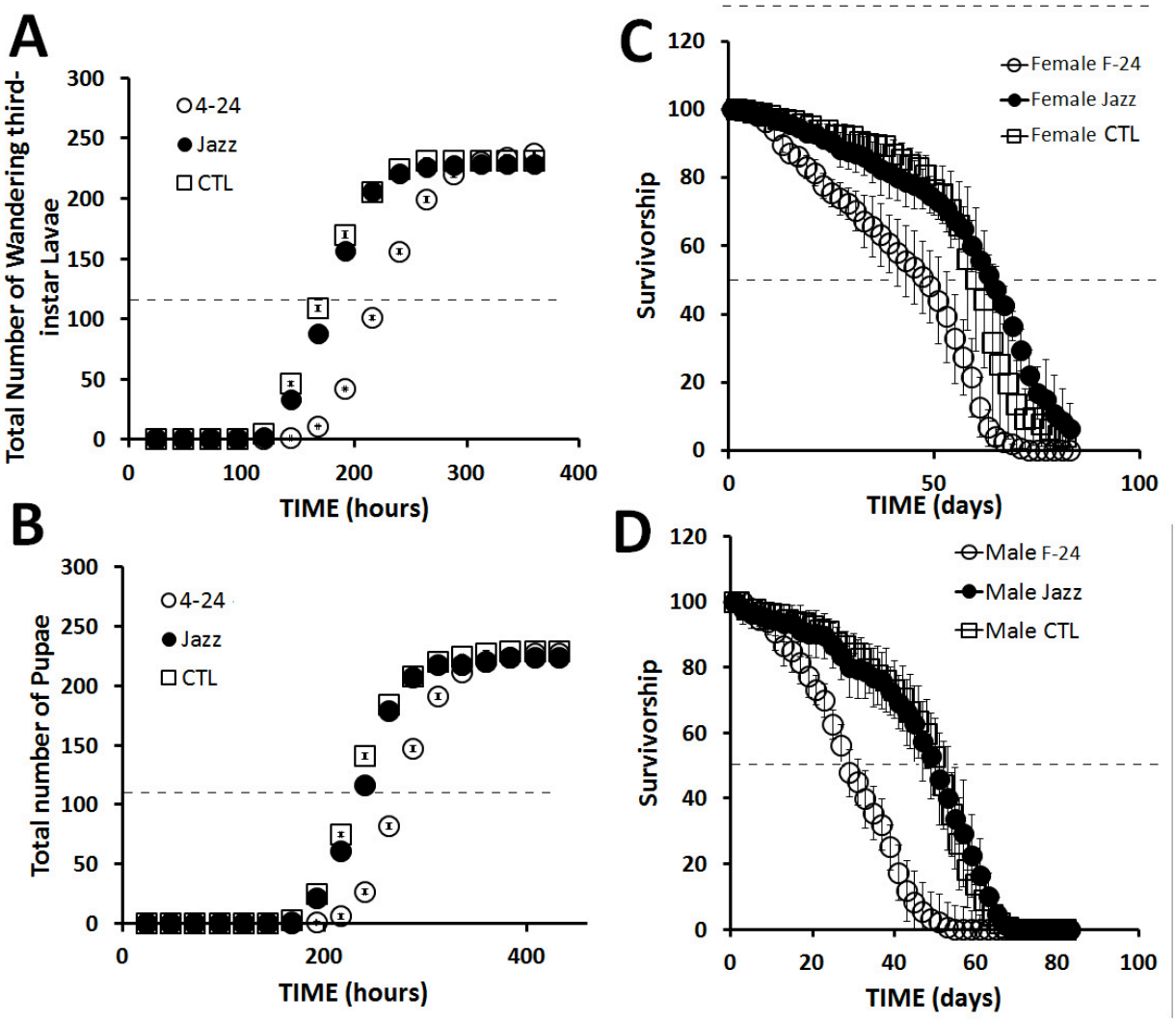
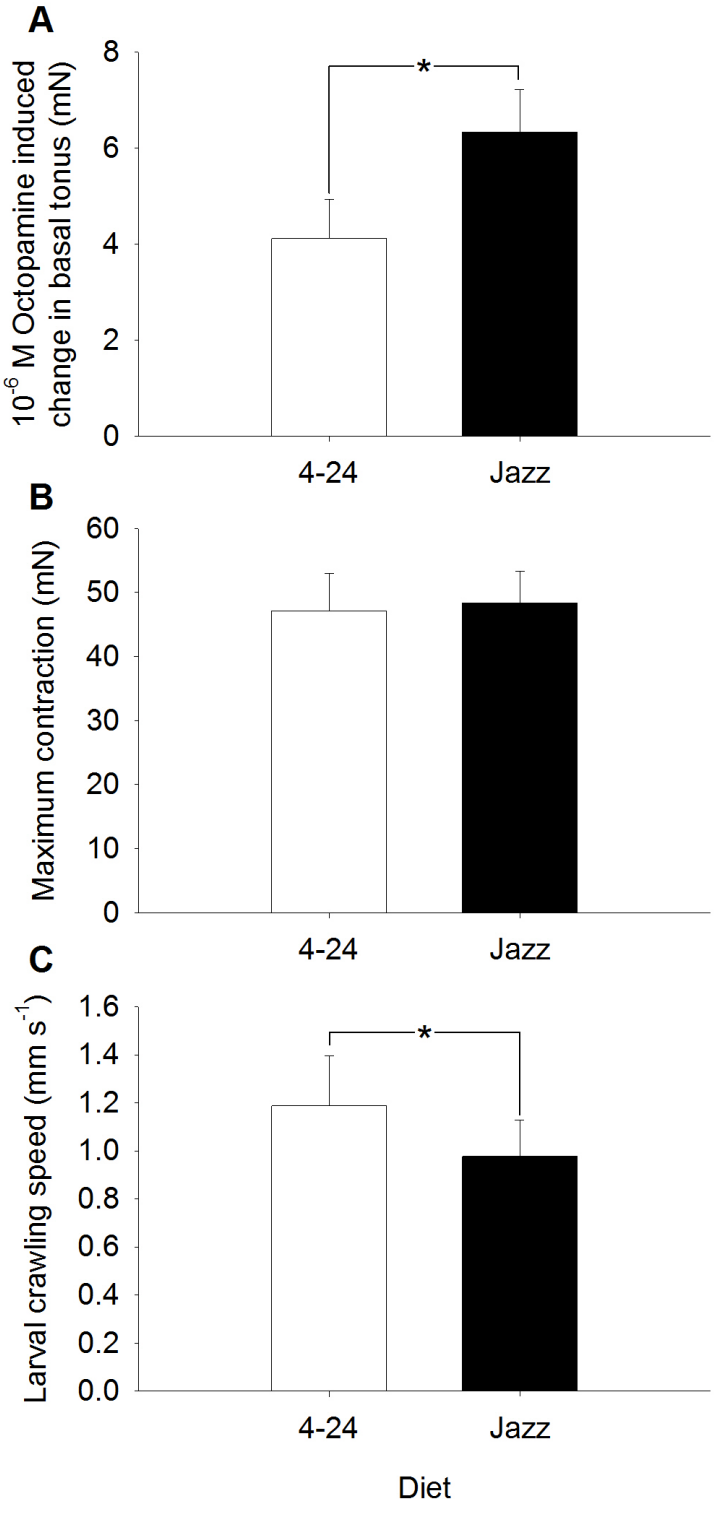


Figure 1: Diet-dependent changes in the average developmental times of: A. larvae, B. pupae, C. Female Adults, D. Male adults. Horizontal dotted lines represent the 50% development and survivorship values (A-C, N=12 vial replicates per diet). 50% values in days (Formula 4-24 vs Jazz-mix vs. Control): Third-instar – 223.6 ± 1.65 vs. *177.2 ± 2.9 vs *170.6 ± 2.8. Pupae – 276.1 ± 2.2 vs. *237.9 ± 2.0 vs *231.7 ± 2.3. Female adults – 47.1 ± 11.1 vs. *63.6 ± vs. 60.0 ± 12.6. Male adults – 29.1 ± 7.1 vs. 49.7 ± 12.3 vs. 50.4 ± 11.3. *indicates a significant effect of diet compared to Formula 4-24 (Mean ± SD, $P<0.05$).



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976 Figure 2: A) 10^{-6} M octopamine-induced changes in basal tonus (*P<0.01, N=10 per diet); B) the
977 force of maximal contraction (elicited by 300 mM KCl) for larvae reared on both diets (N=10 per
978 diet); and C) respective crawling speeds (Mean ± SD, *P<0.05, N=10 per diet).

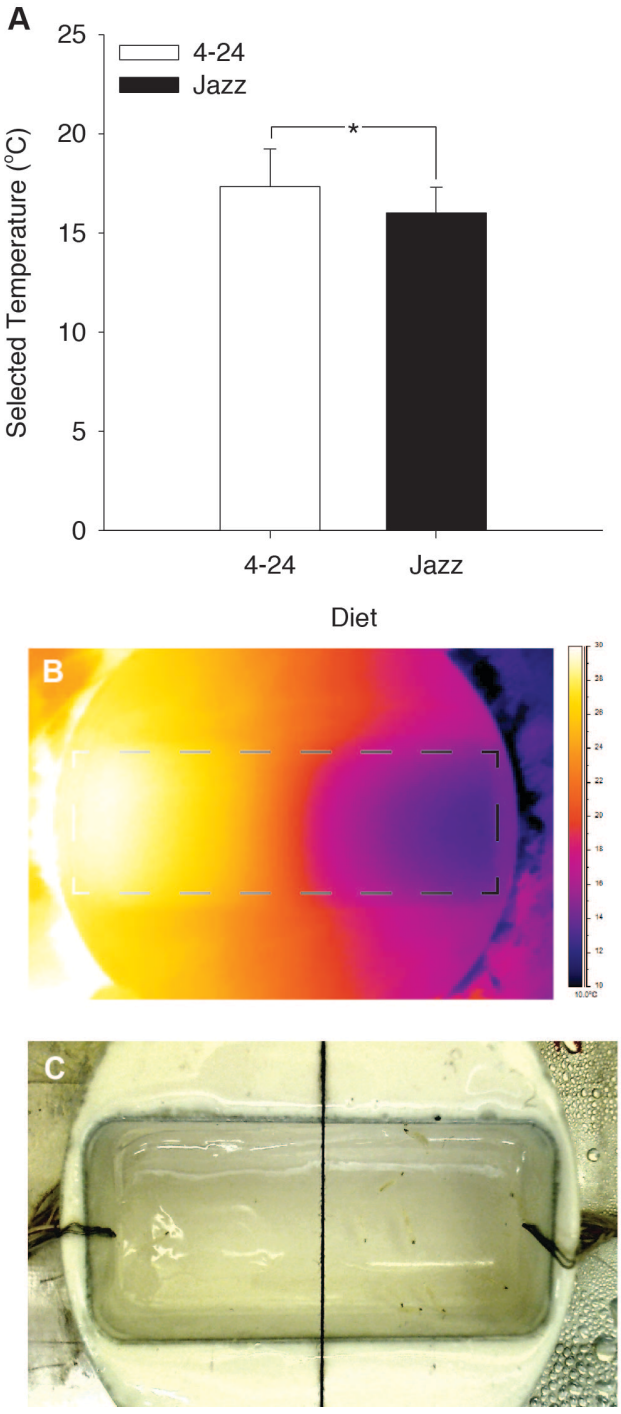


Figure 3: A) Thermotaxis of larval *Drosophila melanogaster* reared on two different commercially available media, tested on a thermal gradient. B) Image of thermal gradient using a thermal camera. C) Image of thermal gradient under ambient light. Larvae reared on Formula 4-24 had a significantly higher preferred temperature compared to those reared on the Jazz-mix (Mean ±SD; 17.32 ± 0.43°C and 15.99 ± 0.29°C; * $P<0.05$; N=20 per diet).

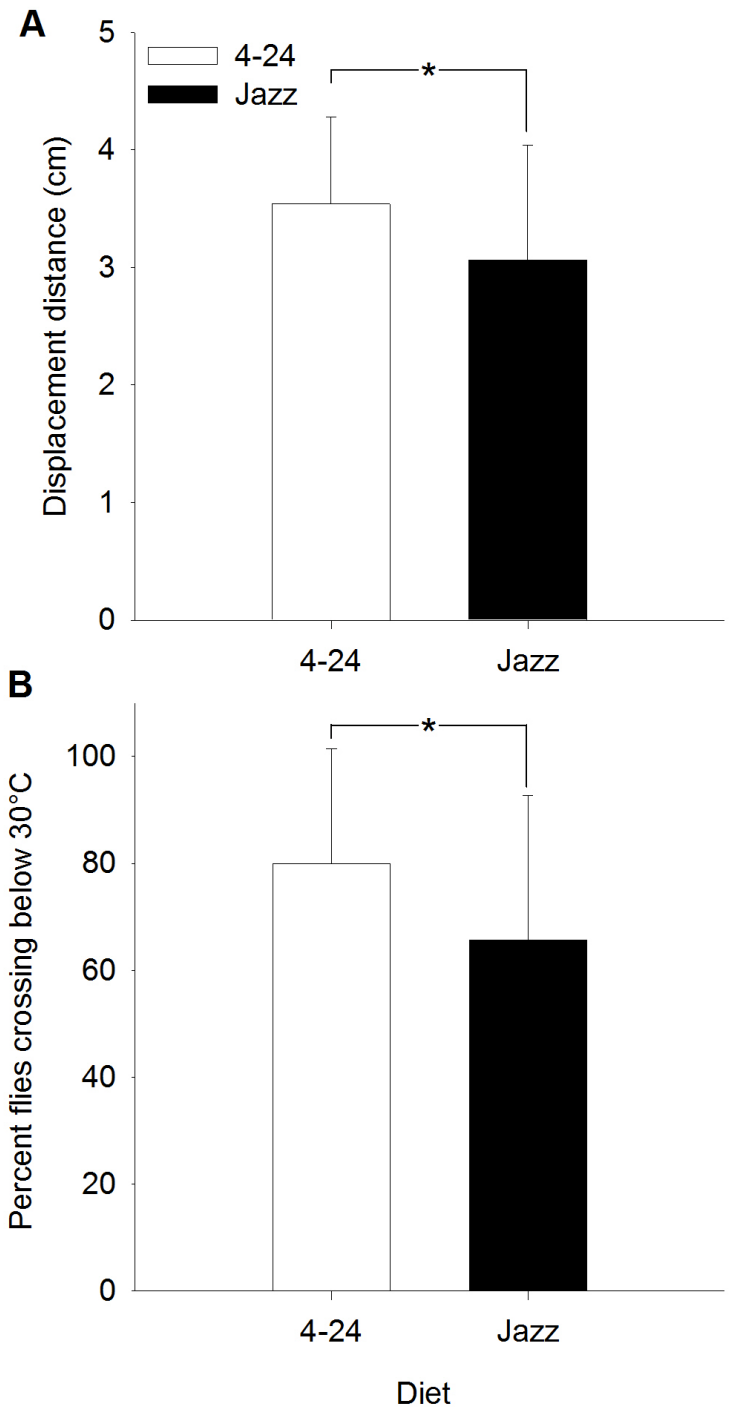
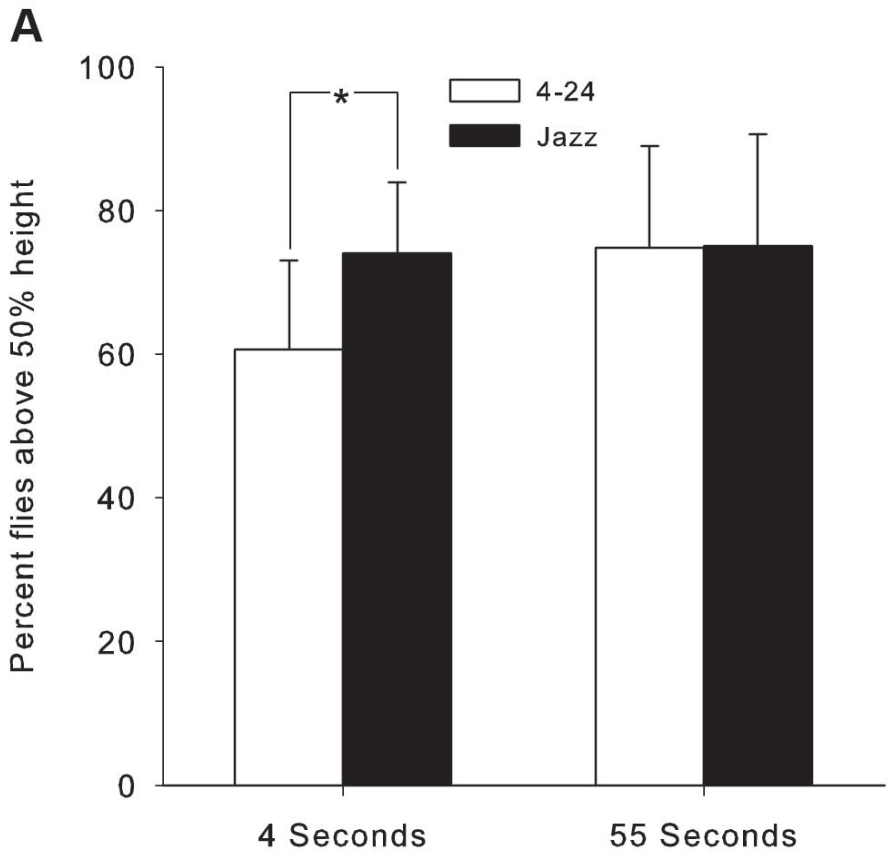


Figure 4: A) Larval *Drosophila* reared on the Formula 4-24 retreated significantly further than those reared on Jazz-mix when placed on the hot side (38°C) of a thermal gradient ranging from 22-38°C (Mean ± SD; $P<0.01$, N=54). B) A greater percentage of larvae reared on Formula 4-24 retreated beyond the 50% or 30°C point than those reared on Jazz-mix (N=54, * $P<0.01$).



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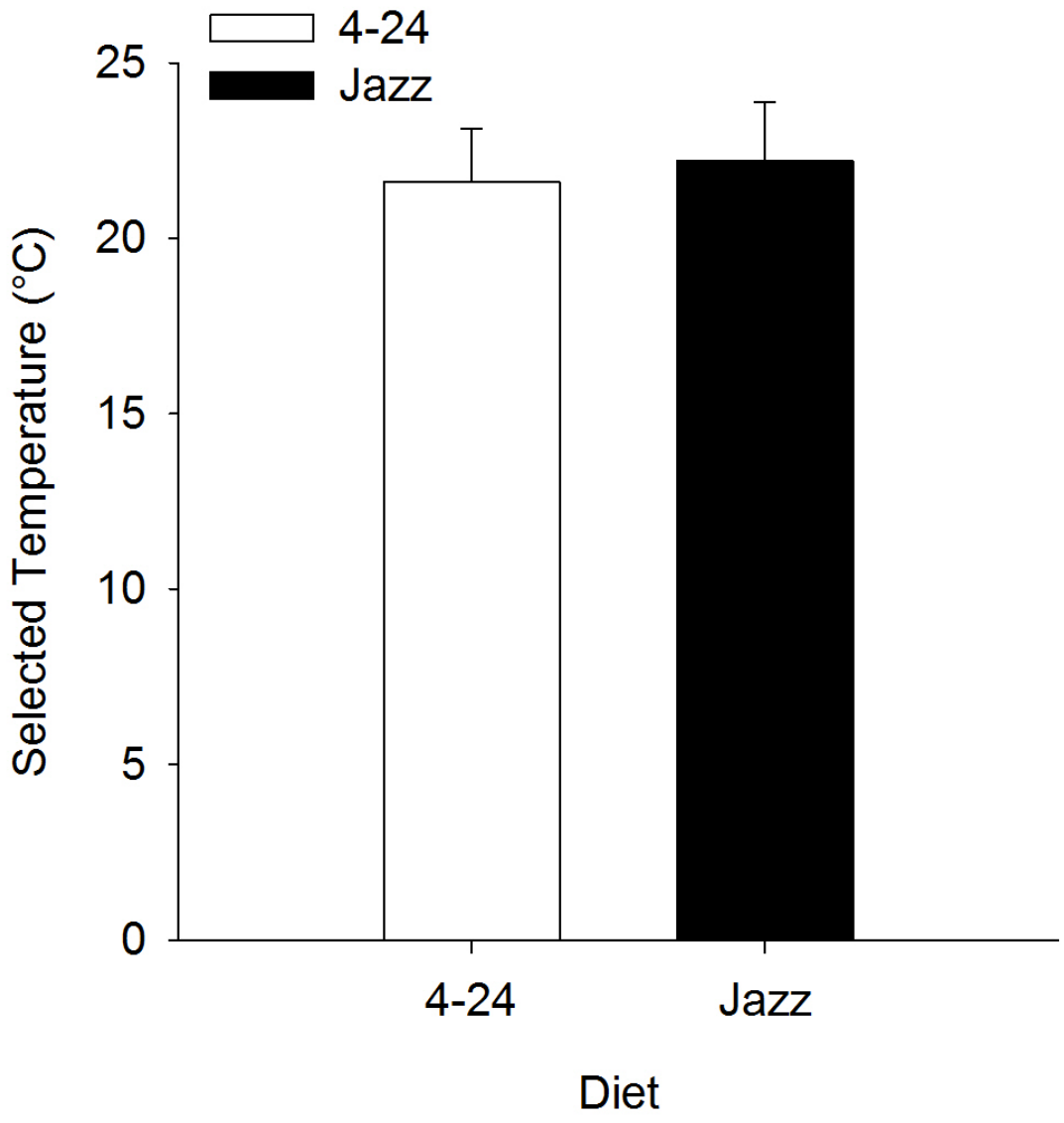
991 Figure 5: A significantly greater percentage of adult *Drosophila* climbed (A) beyond the 50%
992 point when reared on jazz mix than those reared on Formula 4-24 (Mean ± SD; * P<0.01, N=12
993 per diet). B) Depicts the apparatus used to assess negative geotaxis.



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995 Figure 6: Thermal preference in adult *Drosophila* was not influenced by diet (Mean ± SD;
996 P=0.25, Formula 4-24, N=19; Jazz mix, N=17).

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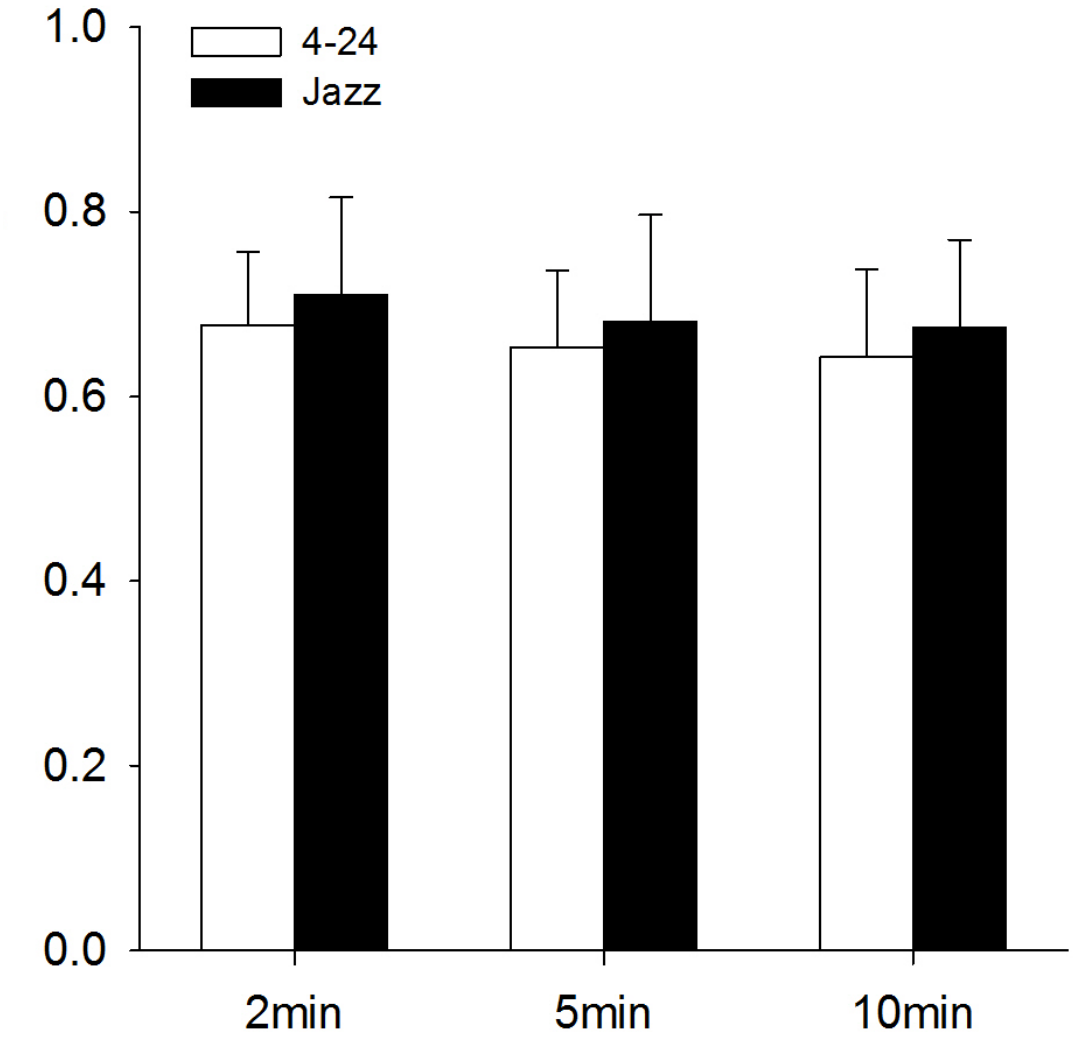

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999 Figure 7: Learning and memory assay reveals that adult *Drosophila melanogaster* learn equally
1000 well irrespective of the diet they are reared upon (Mean ± SD; *P>0.05, N=10).

1001