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### Novel Aspects of *Drosophila suzukii* (Diptera: Drosophilidae) Biology and an Improved Method for Culturing this Invasive Species with a Modified *D. melanogaster* Diet

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**Authors**

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# Novel aspects of *Drosophila suzukii* (Diptera: Drosophilidae) biology and an improved method for culturing this invasive species with a modified *D. melanogaster* diet

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## Abstract

*Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), the spotted wing drosophila, is a global pest of soft fruits now rearable on a standard *D. melanogaster* (Meigen) diet containing the fly's own natural food: soft-skinned berries. The techniques tested here can save 40% of cultures from microbial contamination that develops after combining artificial food sources (e.g., standard drosophila media) with unsterilized host plant material (berries). A suitable ratio for mixing dietary ingredients for a vial or test-tube rearing system includes, by weight, 1 part berry tissue for oviposition, 1.5 parts dry diet media for carbohydrate, 7 parts clean water for moisture, and ~5 grains (0.8 mg) of dry yeast for protein. One or two blackberry or blueberry fruits used by spotted wing drosophila as edible oviposition substrates doubled and tripled pupal and adult production in standard 68 mL culturing vials. To prevent mold from spoiling the diet, the exocarp of berries was sterilized in an 80 or 90% ethanol bath at room temperature for ~5 min, followed by a thorough rinsing with deionized water to remove residual alcohol, which can be acutely toxic to *D. suzukii*, a highly ethanol-intolerant species. Sterilized fruit and a larger fly population in vials disrupted the growth of microbial biofilms capable of suffocating adults. Identical body size in reared adults and locally caught wild flies of *D. suzukii* substantiates nutritional similarity between the fruit-media-based diet and the fly's own natural food (i.e., whole berries). Triethylamine (50%), a common fly anesthetic, was acutely toxic to *D. suzukii* adults but not to *D. melanogaster* adults.

Key Words: spotted wing drosophila; mass rearing; insect diet; invasive pest; ecology

## Resumen

*Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), la drosophila de alas manchadas, es una plaga mundial de frutas blandas que ahora se puede criar sobre una dieta estándar de *D. melanogaster* (Meigen) que contiene alimentos naturales propios de la mosca: bayas con la cascara blanda. Las técnicas de prueba aquí pueden ahorrar un 40% de los cultivos de la contaminación microbiana que se desarrolla después de la combinación de fuentes de alimentos artificiales (por ejemplo, el medio estándar para drosophila) con material de planta hospedera no esterilizada (bayas). Una proporción adecuada para la mezcla de ingredientes de la dieta para un sistema vial o tubo de ensayo de cría incluye, en peso, 1 parte del tejido de baya para la oviposición, 1,5 partes de medio seco de dieta para hidratos de carbono, 7 partes de agua limpia para la humedad, y ~5 granos (0,8 mg) de levadura seca de proteínas. Uno o dos frutos de arándano o moras negras utilizados para la drosophila de ala manchada como sustratos comestibles para oviposición duplicó y triplicó la producción de pupas y adultos en 68 viales estándar de cría mL. Para evitar que el molde no ruine la dieta, se esterilizó el epicarpio de las bayas en un baño de etanol al 80 o 90% a temperatura ambiente durante ~5 min, seguido de un enjuague a fondo con agua desionizada para eliminar el residuo del alcohol, que puede ser muy tóxico para *D. suzukii*, una especie muy intolerante al etanol. La fruta esterilizada y una población de moscas más grandes en viales interrumpieron el crecimiento de biopelículas microbianas capaces de sofocar los adultos. El tamaño del cuerpo idéntico en adultos criados y moscas salvajes *D. suzukii* capturados localmente corrobora la similitud nutricional entre la dieta del fruto/medio y el alimento natural propio de la mosca (bayas enteras). Trietilamina (50%), un anestésico común de moscas, fue muy tóxico para adultos de *D. suzukii*, pero no para los adultos de *D. melanogaster*.

Palabras Clave: drosophila de alas manchadas; cría en masa; dieta del insecto; plaga invasora; ecología

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Spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) is a large vinegar fly that has spread from its center of origin in Asia to Hawaii, North America, and Europe, and is now beginning its invasion of fruit-producing areas of South America and Africa

(Asplen et al. 2015). Currently, crops in Australia and New Zealand show no signs of *D. suzukii* activity. However, these nations' proximity to the home range of *D. suzukii* may soon lead to an invasion by this fly species (Markow & O'Grady 2006; Biosecurity Australia 2010). In

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2008, *D. suzukii* began to inflict 50 to 100% berry losses in parts of the western U.S., primarily in California, Washington, and Oregon (Lee et al. 2011; Cha et al. 2014). Two years later, eastern and southern U.S. states began losing fruit to *D. suzukii*. Four to 5 yr after that, *D. suzukii* damage accounted for an estimated 6% loss in blueberry yield and 34% loss in bramble yield, and with time, these losses are expected to rise because of the increased likelihood of fruit rejections during or after harvest (Price et al. 2009; Goodhue et al. 2011; Burrack 2014).

In exotic habitats, *D. suzukii* has become a major harvest pest of berry crops. The females possess long, sclerotized, and deeply serrated ovipositors capable of inserting eggs into the mesocarp of fresh intact berries (Atallah et al. 2014), a behavior unknown in the common saprophagous species, *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae). Consequently, *D. suzukii*'s niche outside of Asia overlaps little with any other native North American drosophilid, and together with its wide polyphagy (100+ fruit-bearing wild and cultivated plant species from 25 families), has fostered this species' extreme invasiveness (Biosecurity Australia 2010). The females of *D. suzukii* prefer ovipositing into bumpy fleshy fruits with a thin skin, e.g., fruits that have drupes or achenes (blackberries, raspberries, and strawberries), but dozens of wild berry species can serve as alternative hosts for *D. suzukii* to breed almost year-round in the U.S. (Lee et al. 2015; Poyet et al. 2015). Two dozen of *D. suzukii*'s crop hosts yield berries with an exocarp thin enough for ovipositors to puncture. Some of these crops include globally important and high-value fruit species including stone fruits (peaches, cherries, and apricots), blueberries, overripe grapes, citrus, apples, pears, persimmons, figs, and pomegranates (Price et al. 2009; Beers et al. 2011; Stewart et al. 2014).

With any new invasive insect species, the development of optimal diets is crucial for the success of any integrated pest management program and one of the first steps in obtaining adequate live material for study (Vanderzant 1974). Rearing drosophilid flies is well established. However, rearing *D. suzukii* for research is more difficult than culturing *D. melanogaster* or other drosophilids for two reasons. First, *D. suzukii* females preferentially oviposit into firm thin-skinned fruits, not into soft media or overly soft and decomposing berries. Second, and perhaps more importantly, *D. suzukii* cultures lacking solid fruit in their diet are highly susceptible to microbial disease (B. J. Sampson, personal observation). Although *D. suzukii* related articles based on laboratory tests briefly describe diet methodology, none mentions incidences of disease, use of antibiotics, or the contributions of the diet's ingredients to fly nutrition and rearing success. Fruit-amended diets for *D. suzukii* commonly have included purees of fresh fruit, either peach or raspberry, with (Stewart et al. 2014) or without the addition of an agar thickener (Hardin et al. 2015). Whole berries (e.g., raspberries), one or perhaps more, can be dropped into a rearing vial onto the soft diet media (Kacsoh & Schlenke 2012). These authors did not describe the quantities of ingredients or microbial contamination caused by adding or pureeing fruit in the *D. suzukii* diet. These methods also do not mention supplementing the diet media with symbiotic yeasts, which are recommended for use in *D. melanogaster* media to increase fly yield and curb microbial contamination. *Drosophila* species typically vector a community of yeasts, of which many species, once introduced into host tissues, serve as vital sources of nutrients for ovipositing females and developing larvae. The presence of yeast, which can outcompete harmful microbes, can stimulate oviposition in many fruit-breeding species of *Drosophila*, and perhaps yeast benefits reproduction in *D. suzukii* (Hamby et al. 2012).

The rearing method described here is similar to the fruit-based diet described by Kacsoh & Schlenke (2012). However, we discuss problems encountered while rearing locally captured *D. suzukii* and solutions found to optimize this species' diet. We chose *D. melanogaster*

as a control in some of our studies because of its close taxonomic relationship with *D. suzukii* and its ease of management. We tested three hypotheses related to improving the management of captive *D. suzukii* populations. The first hypothesis tested was that incorporating blueberry or blackberry fruit into standard *D. melanogaster* diet media increases *D. suzukii*'s oviposition rate, culture productivity, disease resistance, and adult fitness based on body size (Promislow et al. 1998). Second, inoculating fresh media with dry baker's yeast further promotes *D. suzukii*'s reproductive output. Third, *D. suzukii* adults poorly tolerate the fly anesthetic 50% triethylamine (FlyNap®) when compared with their taxonomic relative *D. melanogaster*.

## Materials and Methods

### FLY STOCKS AND REARING CONDITIONS

*Drosophila suzukii* and *D. melanogaster* flies used in these series of feeding experiments came from our Mississippi cultures, which represented inbred fly cultures (for ~30 generations) originally started with summer morph adults of *D. suzukii* collected from fruit harvested from rabbiteye blueberry plants in 2012 at an experimental farm near Old Creek and Indian Camp Branch, Perkinston, Mississippi (30.7872°N, 88.9906°W). Wildtype *D. melanogaster* flies were captured in 2012 from a fruiting fig tree near Bonner Lake, Poplarville, Mississippi (30.8328°N, 89.512500°W). Inside 68 mL plastic vials for the rearing of *D. suzukii* and *D. melanogaster*, we placed standard formula 4-24® instant media (Carolina Biological Supply, Burlington, North Carolina). However, without berries, microbial contamination contributed to the total loss of approximately one-half of *D. suzukii* cultures. Therefore, we tested protocols to increase rearing success and disease resistance in *D. suzukii*. A series of experiments were set up to test whether the addition of small intact berries (e.g., blueberry or blackberry) can augment reproduction and thwart microbial contamination. Anchoring surface-sterilized berries (fresh or thawed briefly, dipped in ethanol) halfway into the media kept fruit from rolling around and crushing flies. Fly cultures were maintained in standard plastic 68.0 mL drosophila vials kept at 21 to 25 °C under a 24:0 h L:D photoperiod.

### EXPERIMENT 1: INCLUSION OF WHOLE FRUIT IN DIET MEDIA

#### Effects of Diet on Fecundity

The effect of oviposition substrate (fruit species) on early fly fecundity was assessed for female flies ovipositing into or atop blueberry fruit inside small, 8 cm<sup>3</sup>, cube-shaped, plastic chambers ( $n = 243$  blueberries). Three days later, eggs and neonates were counted within the mesocarp or atop the exocarp of blueberry fruits. Turnover rate was calculated as the total number of adults produced after 21 d *minus* the number of parental flies *divided* by the number of parental flies; its mean values for each of the 2 *Drosophila* species were compared with 1-way analysis of variance (ANOVA; GLM procedure of SAS version 9.4; SAS Institute 2013). Chi-squared test compared percentages of older fly cultures continuing to produce adults while feeding for 48, 60, 67, 73, 81, and 101 d on fruit and no-fruit diets ( $n = 115$  cultures).

#### Effects of Diet on Microbial Contamination and Adult Mortality

One hundred and twenty-two *D. suzukii* and 55 *D. melanogaster* cultures were examined for the effects of adding fruit on incidences of microbial infection. Such microbial contamination was evidenced by fuzzy green or whitish masses growing on fruit and, later, by sooty black spores coating the vial's interior as well as by slimy light brown

to orange biofilms that invariably trapped and killed every *D. suzukii* adult in affected vials. The percentages of these cultures showing signs of microbial contamination were then calculated and compared statistically. In Jul 2015, assessments of disease resistance imparted by yeast inoculum were extended to another 54 *D. suzukii* cultures and 55 *D. melanogaster* cultures. Comparative disease resistance in *D. melanogaster* raised on a standard diet without fruit and *D. suzukii* raised on a blackberry/blueberry-based diet was evaluated with Chi-squared tests.

As  $\geq 80\%$  ethanol was the primary means of surface sterilizing berries to prevent microbial contamination, this sterilant's oral toxicity to adults of *D. suzukii* and *D. melanogaster* was gauged by adding 0.0, 1.0, 2.5, 5.0, 7.5, and 10.0% HPLC-grade ethanol to dried diet formula. Six vials containing media at 1 of these 6 ethanol concentrations were arranged in randomized complete blocks. After the ethanol-media diet had fully set,  $\sim 14$  (SE = 0.3) adult flies were transferred to each vial without regard to sex. Rearing temperature for all cultures ranged from 21 to 25 °C and overhead fluorescent lamps provided constant light. Percentage of adult mortality was recorded 24 h after introduction, and the number of dead flies and number of flies in each trial served as dependent variables in probit analyses. Sex-specific sensitivities to dietary ethanol were further tested for 5 flies of a single sex per vial (15 replicates,  $n = 30$  vials) feeding on diet media only (no fruit) containing 10% ethanol, a concentration approximating the LC50 value (9%) for ethanol consumed by *D. suzukii*. Percentage of mortality was then tracked hourly for 6 h. Probit analysis (PROBIT procedure of SAS version 9.4; SAS Institute 2013) with logits modeled the dose–mortality responses of *D. suzukii* and *D. melanogaster* to 6 concentrations of ethanol and the sex-specific differences in adult *D. suzukii* mortality.

### Effects of Diet on Pupal Production

In addition, other host fruit species were tested along with blackberry, a benchmark host (baseline control). These hosts included black cherry, strawberry, blueberry, and white grape. Fly cultures feeding on these specific fruit species (treatments) were arranged in completely randomized blocks. Multiple Wilcoxon 2-sample tests (NPAR1WAY procedure of SAS version 9.4; SAS Institute 2013) compared mean pupal production among diets containing specific fruit species.

### Effects of Diet on Adult Body Size

Body measurements were taken of 80 randomly selected, euthanized (triethylamine overdose) male and female flies collected from wild habitat (blueberry fruit collected in Poplarville, Mississippi) and from healthy, disease-free laboratory cultures raised in Poplarville, Mississippi. Body size served as an index of adult quality and spanned the entire length of the body from the anterior edge of the compound eye to the posterior edge of the terminal abdominal segment. Width of the compound eye at its widest girth served as a second index of body size. Size was used to assess *D. suzukii* growth responses to the presence or absence of dietary fruit tissue. A third body size index was developed, one that was less sensitive to variability in abdominal shape. Accordingly, only the fly's head and thorax was measured from the anterior edge of the compound eye to the posterior-most point of the scutellum. Forty *D. suzukii* adults (20 males from fruit-based cultures and 20 females from no-fruit-based cultures) were measured using the third body size index with an ocular micrometer calibrated at 20 $\times$  magnification under an Olympus SZ60 stereo-zoom dissecting microscope. One-way analysis of variance (ANOVA; GLM procedure of SAS version 9.4; SAS Institute 2013) compared mean body size between *Drosophila* sexes and between fruit- and no-fruit-based diets.

### EXPERIMENT 2: INCLUSION OF A SUPPLEMENTAL YEAST IN DIET

We tested whether inoculating the media with a protein-rich symbiotic yeast (Fleishmann's dry baker's yeast) can improve rearing success of *D. suzukii*, a fly species that uses fresh fruit, a microhabitat largely devoid of natural yeast or other rich protein sources. We compared peak pupal production in those *D. suzukii* cultures raised on diets with or without exogenous baker's yeast. Fly cultures feeding on these 2 diets (treatments) were arranged in completely randomized blocks. Multiple Wilcoxon 2-sample tests (NPAR1WAY procedure of SAS version 9.4; SAS Institute 2013) compared mean pupal production between diets with and without supplemental yeast as a protein proxy.

### EXPERIMENT 3: ADULT ANESTHETIZATION WITH TRIETHYLAMINE

Initially, the standard fly anesthetic, 50% triethylamine (FlyNap®, Carolina Biological Supply Company, Burlington, North Carolina) was observed killing or permanently paralyzing far more *D. suzukii* than *D. melanogaster* flies. Therefore, to corroborate this observation, an absorbent wand was inserted under the foam stopper to dispense triethylamine inside each clean vial containing  $\sim 30$  adults of either *D. suzukii* or *D. melanogaster* for 1 of 5 exposure times: 15, 30, 60, 90, and 120 s. Each of 360 adult flies exposed to triethylamine was regarded as a replicate. The percentage of flies recovering after 195 min was compared across exposure times with Cochran–Armitage trend analyses using the test SMDRC in the FREQ procedure of SAS version 9.4 (SAS Institute 2013).

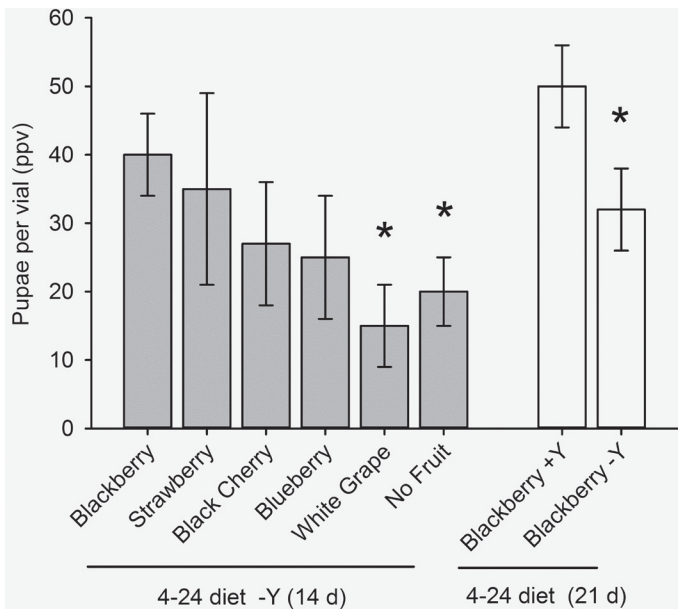
## Results

### INCLUSION OF WHOLE FRUIT IN DIET MEDIA INCREASES CULTURE PRODUCTIVITY AND HEALTH

Fitness of reared *D. suzukii* based on oviposition success and culture productivity varied with the fruit crop chosen as an oviposition substrate. Confined with blueberry fruit, a single mated female laid 10 eggs per berry in 3 d, and 90% of these eggs were inserted fully into the mesocarp (mean  $\pm$  SE =  $3.30 \pm 0.66$  mesocarpic eggs per day,  $n = 243$  berries). The tiny remainder of eggs sat fully exposed atop the fruit surface (mean  $\pm$  SE =  $0.40 \pm 0.08$  eggs per day). The *D. suzukii* females were opportunistic egg-layers, ovipositing upwards of 17 eggs into blueberry picking scars or other deep blemishes.

By addition of fruit to diet media, the probability of mature 21-d-old cultures reaching mean peak pupal production of 73 pupae per vial doubled. Cultures of *D. suzukii* produced equivalent numbers of pupae after 21 d of culture growth, regardless of the fruit species used, or lack thereof (Fig. 1;  $F = 1.08$ ;  $df = 5, 35$ ;  $P > 0.38$ ). However, for 14-d-old *D. suzukii* cultures (Fig. 1) with diets containing a blackberry fruit, pupal yields were comparable to those obtained on diets containing black cherry (1 fruit per vial, Wilcoxon 2-sample test;  $Z = 0.72$ ;  $P > 0.45$ ), blueberry (3 fruits per vial, Wilcoxon 2-sample test;  $Z = 1.52$ ;  $P > 0.05$ ), and strawberry (0.5 fruit per vial, Wilcoxon 2-sample test;  $Z = 0.72$ ;  $P > 0.45$ ). These yields were also twice those of vials with diets containing a white grape (Wilcoxon 2-sample test;  $Z = 2.16$ ;  $P < 0.05$ ) or no fruit (Wilcoxon 2-sample test;  $Z = 2.16$ ;  $P < 0.05$ ).

Adult quality, based on body size, within disease-free *D. suzukii* cultures equaled that of wild-caught adults. Females whether wild or reared had the same body length ( $2.80 \pm 0.04$  mm;  $F = 2.37$ ;  $df = 1, 39$ ;  $P > 0.13$ ) and eye width ( $0.46 \pm 0.01$  mm;  $F = 0.01$ ;  $df = 1, 39$ ;  $P > 0.90$ ). Similarly, reared and wild males were approximately the same size ( $2.22 \pm 0.03$  mm vs.  $2.29 \pm 0.04$  mm;  $F = 2.03$ ;  $df = 1, 36$ ;  $P > 0.16$ ). Reared males, however, had larger eyes or, more precisely, a larger



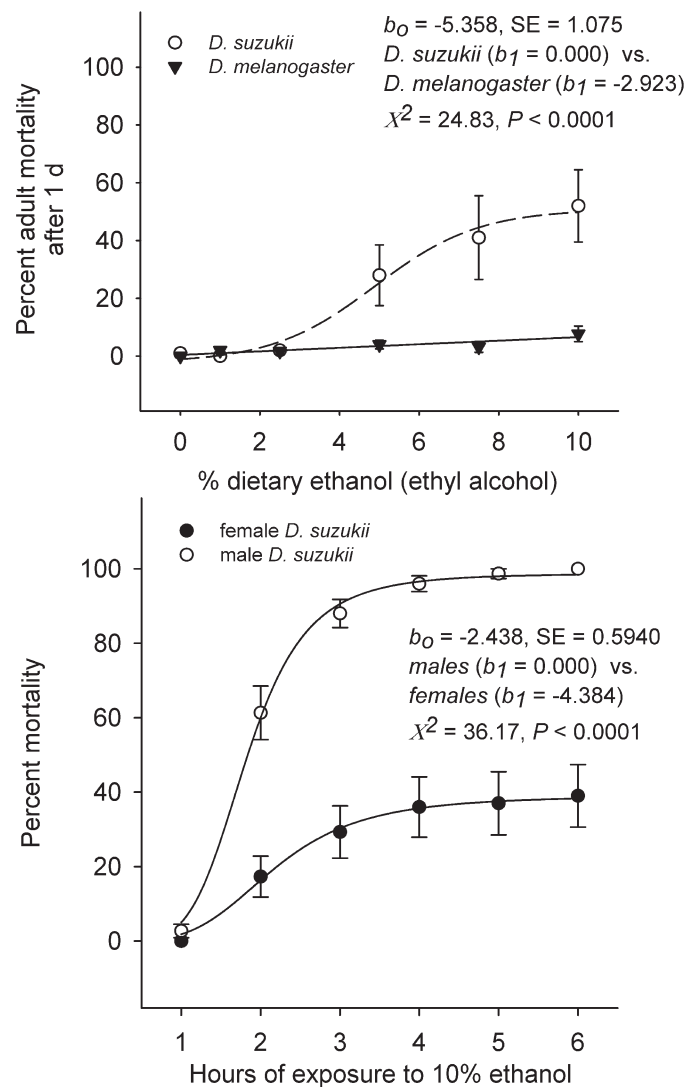
**Fig. 1.** Dietary manipulation of *Drosophila suzukii* cultures based on the use of 5 berry species (blackberry, strawberry, black cherry, blueberry, and grape) with a no-fruit (No Fruit) control (grey bars), and cultures to which yeast was added (+Y) or omitted (-Y) from 4-24® drosophila media (white bars). Asterisks indicate mean differences from 2 respective baseline controls (B), i.e., Blackberry-Y (14 d) and Blackberry+Y (21 d), according to multiple Wilcoxon 2-sample tests.

area of red pigment ( $0.43 \pm 0.01$  mm vs.  $0.37 \pm 0.01$  mm;  $F = 17.07$ ;  $df = 1, 36$ ;  $P < 0.001$ ). For laboratory-reared *D. suzukii*, body size for male and female flies varied little between diets with or without blackberry fruits (fruit vs. no-fruit diet;  $F = 0.00$ ;  $df = 1, 89$ ;  $P > 0.9400$ ). Males were the same size irrespective of diet ( $1.40 \pm 0.02$  mm from anterior of eye to posterior of scutellum) and the same was true for the larger females ( $1.63 \pm 0.02$  mm, male vs. female body length:  $F = 73.45$ ;  $df = 1, 89$ ;  $P < 0.0001$ ).

Without fruit, 2 to 100% (average = 43%) of new *D. suzukii* cultures ( $n = 122$  cultures) contained a fully developed biofilm after 4 to 7 d. Cultures containing a fruit sterilized in 80 to 90% ethanol exhibited 0 to 13% (average 4%) spoilage, a rate similar to that in *D. melanogaster* cultures during the same period ( $n = 55$ ; Chi-squared test;  $P < 0.005$ ). Using deionized water to remove ethanol from sterilized fruit was intended to prevent the inadvertent poisoning of adults of *D. suzukii*, a relatively alcohol-intolerant species (Sampson et al. 2016; Fig. 2). Ethanol concentrations between 0 and 2.5% led to a 6-fold increase in adult turnover after 21 d of culture growth ( $6.11 \pm 0.98$ ) for *D. melanogaster*, twice that of *D. suzukii* (adult turnover rate =  $2.85 \pm 0.57$ ;  $F = 8.55$ ;  $df = 1, 59$ ;  $P < 0.005$ ). In addition, fruit-enriched media prolonged culture productivity in *D. suzukii*; adult production continued within 93% of 48-d-old cultures ( $n = 27$ ), 80% of 73-d-old cultures ( $n = 5$ ), 38% of 81-d-old cultures ( $n = 13$ ), and 29% of 101-d-old cultures ( $n = 7$ ). Sixty percent of cultures without fruit were producing adults after 67 d ( $n = 25$ ). Overall, 39% more mature cultures continued producing adults for 60 to 67 d when diet contained a blueberry or blackberry fruit ( $n = 70$  cultures; Chi-squared test;  $P < 0.05$ ).

#### INCLUSION OF A SUPPLEMENTAL YEAST IN DIET INCREASED CULTURE PRODUCTIVITY

Inoculating media with dry baker's yeast further increased *D. suzukii* pupal production by ~30% (Fig. 1; Wilcoxon 2-sample test;  $Z = 2.00$ ;  $P < 0.05$ ). Moreover, in Jul 2015, *D. suzukii* vials ( $n = 54$ ) containing moistened media lacking yeast and fruit were 98% free of microbial

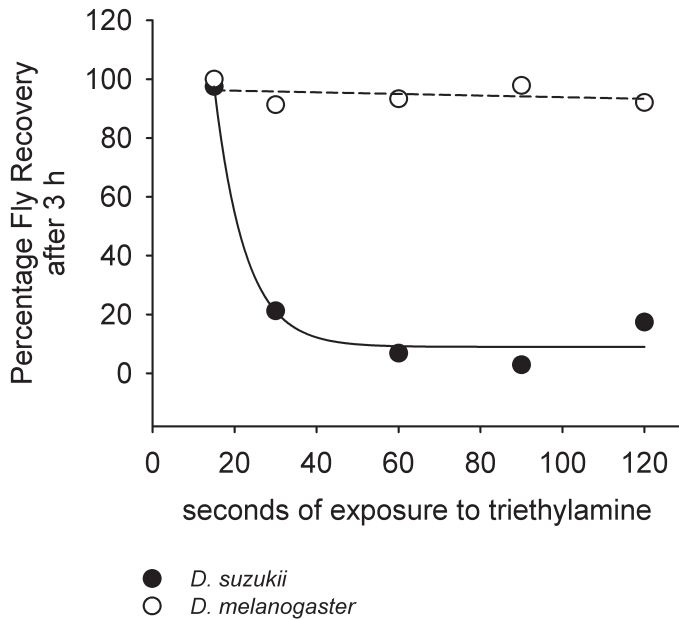


**Fig. 2.** Effects of dietary ethanol (normal environmental ethanol range  $\approx$  9% ethanol) on *Drosophila suzukii* survival when compared with *D. melanogaster* tolerance to ethanol (top graph) and sex-specific sensitivities of *D. suzukii* adults to ethanol (bottom graph). Summary of probit analyses are provided and statistics with  $b_0$  = intercept estimate,  $b_1$  = estimated slope estimate for each fly species, with  $b_1 = 0$  for the baseline control in each graph (*D. suzukii* [top graph], *D. suzukii* males [bottom graph]).

infection, yet, 1 mo later, a random sample of these same vials were found to produce, on average, 33% fewer pupae and adults ( $92.5 \pm 8.2$ ) than did cultures fed a yeast+fruit diet ( $138.7 \pm 8.2$ ;  $F = 13.38$ ;  $df = 18$ ;  $P < 0.005$ ). These latter fruit-enriched cultures were 100% free of microbial contamination ( $n = 44$  vials).

#### ADULT ANESTHETIZATION WITH TRIETHYLAMINE CAN BE ACUTELY TOXIC TO *D. SUZUKII*

Recovery rates of *D. suzukii* adults after exposure to triethylamine for 15, 30, 60, 90, and 120 s averaged 31%, with 20% or less adults recovering after  $\geq 30$  s of exposure (Fig. 3; Cochran–Armitage trend analysis;  $Z = -8.78$ ;  $P < 0.0001$ ). For wildtype *D. melanogaster* adults, overall recovery rate for the same lengths of time was 3-fold higher (94%), varying little despite longer exposures to triethylamine (Fig. 3; Cochran–Armitage trend analysis;  $Z = -0.68$ ;  $P > 0.20$ ). Exposure to triethylamine anesthetic for more than 30 s could kill *D. suzukii* or paralyze



**Fig. 3.** Effect of triethylamine anesthetic exposure on the recovery of *Drosophila* adults: *D. melanogaster* (white circles,  $n = 181$  adults), *D. suzukii* (black circles,  $n = 175$  adults).

70% of adults for more than 3 h. Most immobilized *D. suzukii* adults recovered after 15 s of triethylamine exposure. This short exposure time, however, left some flies active long enough to escape the vials.

## Discussion

A solid food diet for raising laboratory cultures of *D. suzukii* includes some of the same basic ingredients used in other drosophilid diets, e.g., sugar, fruit, and yeast (Dalton et al. 2011; Follett et al. 2014; Jaramillo et al. 2014; Hardin et al. 2015). Although our *D. suzukii* diet is a very simple formulation, the proper ratio of its ingredients is vital for the fly's balanced nutrition. However, the rapid spread of *D. suzukii* and little knowledge of this pest's basic biology and nutrition afforded researchers little time to optimize *D. suzukii* diets. Outbreaks of deadly microbial infections commonly occurred in *D. suzukii* cultures, whether fruit were added or not. Mold commonly originated from fruit but was effectively controlled after fruit surfaces were sterilized with ethanol. These chemically sterilized fruit, however, were highly toxic to flies and, therefore, required pre-washing in sterile water before their addition to diet media.

The *D. suzukii* cultures reached peak pupal production when started with ~20 adults and provided with a carbohydrate-rich diet containing 7 parts clean water to 1.5 parts dry diet to which was added 1 part fruit (w/w/w). Age structure for healthy populations of cultured *D. suzukii* and *D. melanogaster* appeared stable with 2 pupae produced for every emerging adult (Emiljanowicz et al. 2014). Inoculating culture media with dry baker's yeast further increased *D. suzukii* adult and pupal production by one-third. In two other studies, yeast augmentation increased *D. suzukii* oviposition and pupation by a similar rate (Jaramillo et al. 2014; Hardin et al. 2015), indicating yeasts are symbiotic and probably provide *D. suzukii* larvae with the protein that their natural habitats (e.g., ripe or ripening berries) lack (Hamby et al. 2012; Hardin et al. 2015). However, adding far more than 5 grains (>0.80 mg) of exogenous yeast to the diet could harm cultures if fermentation produces toxic levels of carbon dioxide that sterilize, incapacitate, or kill

flies (Markow & O'Grady 2006). Moreover, the diverse fungal community associated with *D. suzukii* is a possible source of endosymbionts that might someday be cultivated and introduced to a rearing system to enhance fly fitness or exploited in novel ways to reduce fly survival and reproduction (Hamby et al. 2012).

In *D. melanogaster* cultures, artificial media alone induces prompt egg laying. In *D. suzukii* cultures, earlier oviposition occurs on natural food substrates, i.e., intact thin-skinned fruits, a discovery made by Kacsoh & Schlenke (2012), who added thawed, frozen raspberries to standard drosophila diet media. Our findings concur with those of Kacsoh & Schlenke (2012); adding intact fruit to drosophila media prompted earlier oviposition by *D. suzukii*. These researchers, however, reported no concomitant increase in overall fly production. In contrast, our fruit-based diet enhanced fly output by (1) hastening larval survival, (2) increasing pupal production, and (3) preventing outbreaks of disease in the cultures. Interestingly, cultures of *D. suzukii* supplied with fruit experienced faster population growth without incurring a concomitant reduction in adult body size. This result suggests cultures had unlimited food resources for the first month of culture growth. Fruit added to diet media probably provides *D. suzukii* adults and larvae with additional macronutrients, micronutrients (e.g., phytosterols), and carbohydrate (Jaramillo et al. 2014; Hardin et al. 2015). Fruit composed 40% of the diet by weight and accounted for a one-third increase in early pupal output. Fortuitously, the addition of whole berries to the drosophila diet controlled disease, which not only saved cultures from destruction, but also enhanced culture productivity by 30%.

Our observation of *D. suzukii* females ovipositing into blueberry picking scars indicates that this species has the capacity to expand its host choice to damaged fruit of host plants and perhaps non-host plants. For instance, picking scars and other forms of harvest damage to non-host fruits such as peaches can induce *D. suzukii* females to lay 4 times more eggs than insect damage does and 10 times more eggs than simulated damage does (Stewart et al. 2014). Hence, fruit marred from hail and during harvest might possibly increase market losses to *D. suzukii* infestation. Therefore, reducing *D. suzukii* population size and fruit injury during harvest could lessen overall yield loss.

Not all intact fruits species stimulate *D. suzukii* oviposition. For instance, white grapes produced no more pupae that did the no-fruit diet. Likewise, muscadine grapes have skins too thick for *D. suzukii* females to oviposit. When given no alternative hosts, *D. suzukii* females misplace or abort their eggs by positioning them horizontally atop un-punctured unblemished muscadine fruits (B. J. Sampson, personal observation). These misplaced eggs withered without larval hatch (Stewart et al. 2014). *Drosophila suzukii* females can easily lay eggs in blueberry and blackberry fruits, two hosts with thin skins. Interesting, this is perhaps the first study that describes females misplacing or aborting a small fraction of their eggs on blueberry, a known cultivated host of *D. suzukii*. This behavior suggests blueberry is a less preferred reproductive host, and does explain lower mean yield losses for blueberry when compared with other cultivated small fruits, notably brambles (Burrack 2014). Successful oviposition occurs only after the female vertically inserts eggs into the mesocarp with their paired respiratory (oxygen) filaments projecting above the puncture site (Stewart et al. 2014). Hence, the ratio of viable eggs (successful oviposits) to aborted or misplaced eggs might serve as an excellent index for measuring host plant resistance.

Berries added to diet media stimulates captive *D. suzukii* females to oviposit, yet in their raw state, fruits can greatly increase the probability of fungal disease (mold). To prevent such fungal infections, the exocarp of whole berries or fruit chunks, whether fresh or thawed, require chemical sterilization with ethanol. Triple rinsing berries with deionized or distilled water then removes or dilutes ethanol to a safe



level and prevents the inadvertent poisoning of alcohol-sensitive adults (Sampson et al. 2015). In their study of *D. suzukii* associated yeasts, Hamby et al. (2012) pre-sterilized flies with 70% ethanol to remove any cuticular yeasts. Afterward, they rinsed away any residual alcohol that, if swallowed, could harm microbial endosymbionts. This prudent pre-washing of flies probably prevented mass death among their experimental flies.

Ethanol has higher acute oral toxicity to *D. suzukii* when compared with saprophagous species like *D. melanogaster* (Sampson et al. 2015). The LC50 value for *D. suzukii* adults imbibing ethanol was ~9%. Adults of *D. melanogaster* after drinking 10% alcohol experienced no apparent mortality and ethanol's LC50 value for this species was twice that observed for *D. suzukii* (Sampson et al. 2015). Detoxification of low concentrations of ethanol ( $\leq 2.5\%$ ) by dehydrogenase enzymes produces acetate, a metabolite used by *D. melanogaster* as a supplemental high-energy food source to double pupal production as well as increase the rate of reproduction to a level twice that of *D. suzukii* (Sampson et al. 2015). *Drosophila suzukii* cannot convert low levels of ethanol to food, suggesting a very low activity of alcohol detoxification. Increasing rates of environmental ethanol destabilized sex ratios of *D. suzukii*, whereas sex ratios of *D. melanogaster* remained unaffected even at dietary ethanol concentrations as high as 40% (Sampson et al. 2015). A female-biased sex ratio for *D. suzukii* cultures probably results from the greater sensitivity of males to dietary ethanol. Without dietary alcohol, the sex ratio for *D. suzukii* stabilized near that of *D. melanogaster*, ~1:1. Hence, using fruit and yeast to rear *D. suzukii* could produce sufficient dietary ethanol to depress the size of male populations without reducing overall culture longevity. Intolerance to higher concentrations of ethanol perhaps evolved in *D. suzukii*, as adult and larval habitats contain little if any environmental alcohol.

Berries sitting in media can inhibit microbial growth by enticing *D. suzukii* oviposition, which in turn may further inoculate food with competing symbiotic microbes (Bergomaz & Boppré 1986), disrupt biofilm formation through larval feeding (Jefferson 2004), and acidify media via fruit decomposition. Small *D. suzukii* cultures with less than 20 adults and lacking a fruit for oviposition are prone to microbial infection and biofilm formation. These sticky biofilms invariably kill every adult fly in a culture. Whether microbial communities associated with *D. suzukii* exude these biofilms is unknown. Clearly, sugar-rich media can spur growth in some harmful microbial species (Chandler et al. 2014). If the presence of a fruit spurs *D. suzukii* eggs to be laid and larvae to hatch within 3 d of culture installation (parents introduced to fresh media), then resulting larvae should have ample time to feed and bury harmful microbes as they move throughout the food matrix. Hence, adding a blueberry or blackberry confers to *D. suzukii* flies resistance to microbial contamination of their food. Likewise, cleaning metal worktables with 100% ethanol and adding ~300  $\mu\text{L}$  of penicillin-streptomycin antibiotic to the media further curbs microbial outbreaks. We have used this antibiotic mixture in the spring months when some microbial outbreaks seem more frequent, especially those outbreaks associated with deadly biofilm formation. However, antibiotics are only effective until biofilm-producing microbes develop resistance to them (Jefferson 2004). Adding fruit to the diet and pre-sterilizing drosophila vials overnight in 5% or 10% bleach solution are more cost-effective measures for suppressing microbial growth. Further microbial control is possible by cleaning foam sponge stoppers (sponges) in soapy water, not bleach, as bleach reacts chemically with foam to darken, shrink, and reduce stopper elasticity.

Adult flies and pupae at risk of drowning in soggy media might benefit from fruit in another way; berries can serve as edible perches. Generally, *D. suzukii* prefers a firmer diet (not dry) to a soggy diet. Therefore, the proper balancing of water-to-diet ratios would depend

on humidity, temperature, fruit size, and berry species. Excessive residual water leaking from decomposing fruit could combine with condensation inside small vials to create droplets or pools large enough to drown flies individually or en masse. However, flies reared in standard (68 mL) plastic vials faced no serious moisture buildup. Fruit degradation seems to preserve media moisture and fly production for approximately 60 to 80 d.

Adults will require immobilization before being examined. *Drosophila suzukii* showed greater sensitivity to triethylamine anesthetic than did *D. melanogaster*. The *D. suzukii* adults could safely tolerate approximately 15 s of exposure to triethylamine; any exposure longer than 15 s caused fly recovery rates to drop dramatically. However, 15 s might be too brief of an exposure to immobilize every fly in a vial. *Drosophila suzukii* and mosquito adults respond similarly to FlyNap® and both species are highly sensitive to this anesthetic (Chen & Hillyer 2013).

Reared and wild *D. suzukii* adults raised on a fruit-based diet were similar in quality based on body size. The relatively larger eyes of reared males perhaps occurred because eyes of wild-caught flies lost some of their ocular pigment to leaching into the preservation fluid. In fact, Jaramillo et al. (2014) assessed body size for *D. suzukii* fed a diet containing either fruit or yeast and discovered that mean body size for males and females remained unchanged.

Current *Drosophila* rearing technology can now be used to prepare a disease-free *D. suzukii* diet. A suitable ratio for mixing diet ingredients was, by weight, 1 part berry for oviposition, 1.5 parts dry diet media for carbohydrate, 7 parts clean water for moisture, and ~5 grains of dry yeast for protein. In 68 mL vials, these proportions of ingredients translate into 2.5 g fresh or thawed blackberries (preferred) or blueberries, 3.75 g dry diet media, 20.0 mL water, and 0.8 mg of dry baker's yeast. Protein-rich yeasts and nutrient-rich fruits are important dietary supplements for *D. suzukii*, a drosophilid species whose larval niche is initially devoid of protein and large cultures of symbiotic microflora. Fruits require sterilization with doses of ethanol deadly to *D. suzukii* (~80–90%) followed by its thorough removal before introducing *D. suzukii* adults. The use of chemical anesthetics such as triethylamine to knockdown adults can be extremely toxic to these flies.

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