Female oviposition decisions are influenced by the microbial environment

Emily K. Fowler[1]*, Lucy A. Friend[1], Emily R. Churchill[2], Douglas W. Yu.[1], Marco Archetti[3], Andrew F.G. Bourke[1], Amanda Bretman[2], and Tracey Chapman[1]*

* Corresponding authors

1. School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

2. School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK

3. Department of Biology, Pennsylvania State University, University Park, PA 16802, USA

Corresponding author(s) contact

Emily Fowler: e.fowler@uea.ac.uk

Tracey Chapman: tracey.chapman@uea.ac.uk

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Conflict of Interest

We declare we have no conflict of interests.

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Author Contributions

Conceptualization: Emily K. Fowler, Lucy A. Friend, Emily R. Churchill, Douglas W. Yu, Marco Archetti, Andrew F.G. Bourke, Amanda Bretman, Tracey Chapman. *Data curation*: EKF. *Formal analysis*: EKF. *Funding acquisition*: EKF, DWY, MA, AFGB, AB, TC. *Investigation*: EKF, LAF. *Methodology*: EKF, LAF, TC, AB. *Project administration*: EKF, LAF, ERC, AB, TC. *Resources*: TC. *Visualization*: EKF. *Writing – original draft*: EKF. *Writing – review & editing*: EKF, LAF, ERC, DWY, MA, AFGB, AB, TC. Our study has co-authors now based in different countries. All authors were engaged throughout the research process to ensure that the diverse sets of perspectives they represent was considered. We used unbiased methods for literature searching during the scoping and planning of the study and those searches formed the basis of the literature cited in the paper. All authors gave final approval for publication.

Data availability

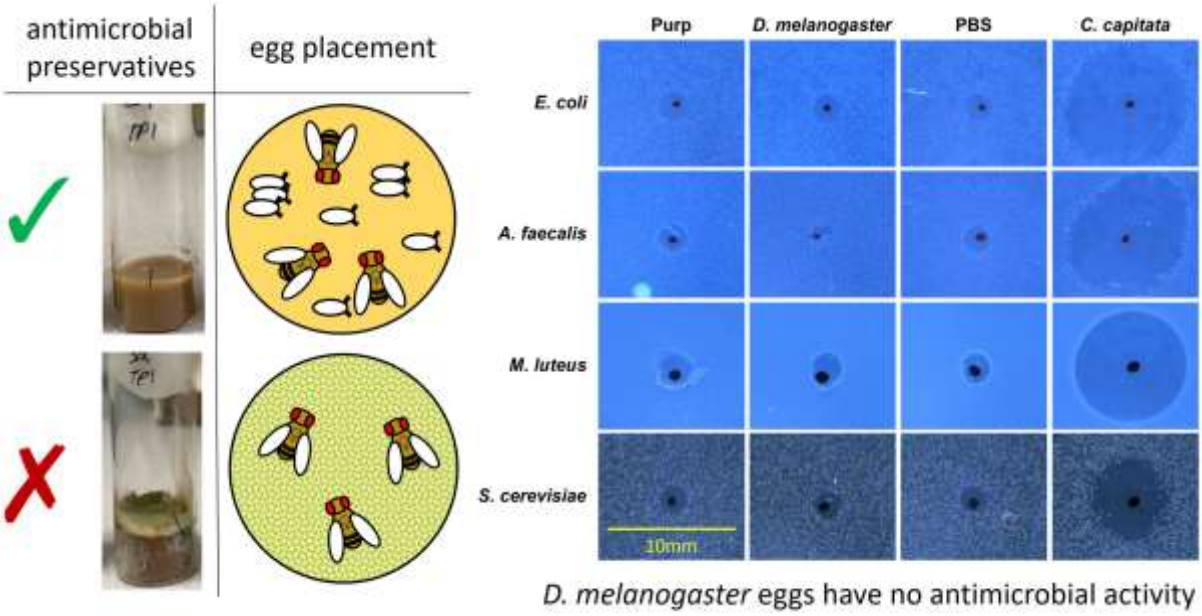
Data and code are available through Dryad (doi:10.5061/dryad.2rbnzs7xk)

Abstract

In ovipositing animals, egg placement decisions can be key determinants of offspring survival. One oviposition strategy reported across taxa is laying eggs in clusters. In some species, mothers provision eggs with diffusible defence compounds, such as antimicrobials, raising the possibility of public good benefits arising from egg clustering. Here we report that *Drosophila melanogaster* females frequently lay eggs in mixed maternity clusters. We tested two hypotheses for potential drivers of this oviposition behaviour: (i) the microbial environment affects fecundity and egg placement in groups of females; (ii) eggs exhibit antimicrobial activity. The results partially supported the first hypothesis. Females reduced egg laying, but did not alter egg clustering, on non-sterile substrates that had been naturally colonised with microbes from the environment. However, oviposition remained unaffected when the substrate community consisted of commensal (fly-associated) microbes. The second hypothesis was not supported. There was no evidence of antimicrobial activity, either in whole eggs or in soluble egg surface material. In conclusion, while we found no behavioural or physiological evidence that egg clustering decisions are shaped by the opportunity to share antimicrobials, females are sensitive to their microbial environment and can adjust egg laying rates accordingly.

Keywords: *Drosophila melanogaster*, microbes, antimicrobial, preservatives, egg clustering, public goods

Graphical Abstract



Introduction

For an oviparous animal, deciding where and how to place eggs can have major fitness consequences (1). Several hypotheses have been proposed for oviposition site selection and, of these, maximising embryo survival is viewed as a key driver (2). As such, females of many species exhibit a remarkable ability to detect and respond to a range of abiotic and biotic conditions when making egg placement decisions. For example, in the tree-hole breeding frog (*Phrynobatrachus guineensis*) females prefer to oviposit at sites already inhabited by conspecific eggs and tadpoles, which is thought to reduce predation risk to their offspring (3). Females also select sites with the appropriate level of water persistence required for successful offspring development. In the pine sawfly *(Neodiprion sertifer*) females prefer to oviposit on trees with high resin acid concentrations, which lowers the vulnerability to attack by parasitoids (4).

Animals can also exhibit oviposition decisions *within* a single oviposition patch or substrate, by adjusting the number of eggs they lay, delaying oviposition if the substrate or environmental conditions are perceived as sub-optimal, or positioning their eggs in non-random patterns (5). For example, females can lay their eggs in dispersed patterns, or cluster their eggs together or with those of other females. Egg clustering behaviour, including mixed-maternity clustering, has been reported for many taxa, including in reptiles and amphibians (6), birds (7), fish (8) and several invertebrate species (e.g. Refs 9, 10)). Egg clustering has recently been studied in the fruit fly *Drosophila melanogaster* and has been shown to be a plastic behaviour that increases in frequency with increasing social density (11).

Several fitness benefits of egg clustering have been proposed, although empirical evidence remains scant (12). For example, egg clustering could be the outcome of females reducing site and substrate evaluation times and instead relying on the decisions of others (10). Alternatively, clustering could reduce egg predation risk if predators have limited search, consumption times or searching capacity. For instance, *Iphiseius degenerans* mites oviposit in clusters in acarodomatial leaf hairs. Females prefer to cluster their eggs in acarodomatia already containing eggs, and clustered eggs are

less likely to be predated by thrips (9). Egg clustering may also increase egg survival during exposure to abiotic factors, such as low humidity. For example, in the Nymphalid butterfly *Chlosyne lacinia*, hatching success is positively related to humidity, and eggs clustered in larger groups have greater desiccation resistance in comparison to small groups of monolayered eggs (13).

In this study, we propose and test an additional hypothesis - that clustered eggs benefit from the collective increased concentrations of defensive (i.e. antimicrobial) compounds potentially provisioned to the egg surface by the mother (14). When such defensive compounds are external and diffusible, they are potential 'public goods', because eggs lacking these defences can nevertheless receive protection from those released by nearby eggs (15). Findings in some fly species suggest this is possible. For example, Mediterranean fruitfly (*Ceratitis capitata*) females smear the surface of their eggs with secretions containing ceratotoxins - a family of broad-acting antimicrobial peptides (AMPs), that are produced in the female reproductive tract (16). The genes encoding ceratotoxins have no known homologues outside *Ceratitis* (17). However, some AMPs of *Drosophila* are similarly expressed in the female reproductive tract and have the potential to be transferred to egg surfaces. For example, the anti-fungal peptide encoding gene *Drosomycin* is expressed in the reproductive tract epithelium (18, 19) and the anti-bacterial encoding gene *Drosocin* is constitutively expressed in the female oviduct (19, 20). The promoters of AMP genes *cecropin*, *defensin*, *metchnikowin* and *attacin* are also active in the reproductive tract (19). A transcriptomic study of female reproductive tissues found that some AMP genes were upregulated following mating (21). It is not yet known why, but one possibility is that it enables the production of AMPs to protect the elevated numbers of eggs that are produced and laid following mating.

Consistent with the idea that egg clustering may facilitate beneficial interactions with microbes or protect against harmful ones, several studies provide evidence that the microbial environment influences oviposition behaviour. For example, when offered a direct choice between substrates containing commensal microbes (i.e. members of the fly-associated microbiome) vs. sterile substrates, *D. melanogaster* prefer to lay on microbe-inoculated substrates, whereas *D. suzukii* prefers sterile substrates (22). These differences may reflect the natural oviposition substrates of these two

species, with *D. melanogaster* laying into fermenting fruit and *D. suzukii* into ripening fruit. The Oriental fruitfly *Bactrocera dorsalis* uses a volatile compound associated with the presence of egg-surface bacteria to avoid laying into fruits already occupied by conspecific eggs (23). There is also evidence that *D. melanogaster* uses sucrose levels as a means of assessing the presence or level of commensal bacteria in their food, since the lactic acid bacteria *Enterococci* metabolises and therefore depletes sucrose within food sources (24). *D. melanogaster* eggs also appear to be dependent on microbes for successful development, with germ-free eggs failing to develop beyond the second instar larvae when reared in food lacking yeast (24, 25).

D. melanogaster females lay eggs in decomposing fruit with a rich microbial environment that is very likely to contain a mix of beneficial, neutral and pathogenic microbial species (26, 27). Although few extracellular pathogens have so far been identified as attacking *D. melanogaster* eggs (27), ingestion of some bacterial strains by larvae can be fatal (26). This suggests there should be selection for choosing or maintaining pathogen-free oviposition sites. Consistent with this, *D. melanogaster* females can detect and avoid the odorous compound geosmin, which is produced by some microbes, including pathogenic species (27). Collectively, these data support the hypothesis that female flies choose oviposition sites according to the prevailing microbial milieu and/or protect their eggs from pathogens by deploying antimicrobials. The latter raises the possibility that oviposition clustering decisions are shaped by potential public good benefits for antimicrobial protection. The aims of this study were to investigate these ideas by testing the hypotheses that: (1) *D. melanogaster* females plastically adjust egg placement based on the microbial environment; and (2) *D. melanogaster* eggs exhibit broad spectrum antimicrobial activity.

Materials and Methods

Fly stocks and handling

Wild type *D. melanogaster* (strain: Dahomey) and a *scarlet* line (backcrossed multiple times into the Dahomey genetic background) were both maintained in large stock cages with overlapping generations. Flies were reared on standard sugar yeast agar (SYA) medium (100 g brewer's yeast (*MP*

Biomedicals, *Fisher Scientific #11425722*), 50 g white caster sugar (*Tate & Lyle*), 15 g agar (*Formedium #AGA01*), 30 ml Nipagin (methylparaben, 10% w/v solution, dissolved in 95% Ethanol), and 3 ml propionic acid (*Sigma-Aldrich #P5561*), per litre of medium) in a controlled environment (25°C, 50% humidity, 12:12 hour light:dark cycle). Eggs were collected from population cages on grape juice agar plates (50 g agar, 600 ml red grape juice (medium dry red wine kit, *Magnum*), 42 ml 10% w/v Nipagin solution per 1.1 l RO H_2O) supplemented with fresh yeast paste (Saf-levure active dry yeast, *Lesaffre*), and first instar larvae were transferred to SYA medium at a standard density of 100 per vial (glass, 75 × 25 mm, each containing 7 ml medium). Male and female adults were separated within 6 hours of eclosion (before mating occurs) under ice anaesthesia and stored in single sex groups of 10/vial until required.

Statistical methods – general principles

All statistical analyses were conducted using R version 4.2.1 (28). Graphs were produced using *ggplot2* (29) and *ggpubr* (30) packages. Summary statistics were produced using the *Rmisc* package (31).

We defined an egg cluster as a group of two or more eggs where any part of the main body of an egg was in physical contact with any part of the main body of another egg (Figure S1). Egg clustering proportion was calculated for vials containing ≥ 2 eggs. Clustering proportion per vial was calculated by summing the total number of eggs in each cluster and dividing that by the total number of eggs laid in the vial (Figure S1) (11). For all analyses, full models containing all explanatory variables and their interactions were fitted in the first instance. Non-significant interactions (as tested using the anova function) were then removed from the models using a stepwise process. Model residuals were plotted and checked visually, using the DHARMa package where possible (32). Hurdle models were used to analyse zero-inflated count data. Negative binomial GLMs were used for over-dispersed count data, and quasibinomial GLMs were used to analyse over-dispersed proportion data. Further details of specific analyses for each experiment are described in the sections below, and the final model outputs are presented in the supplementary material.

Hypothesis 1. *D. melanogaster* females plastically adjust egg placement based on the microbial environment.

We conducted a set of three experiments in which we measured fecundity, egg clustering and egg-adult survival in groups of females in the presence of a range of microbial conditions. Each experiment had a different set of oviposition substrates but was otherwise carried out using the same protocol. Unmated females were collected as described above and then transferred to SYA vials in same-sex groups of four at ~4 days post-eclosion. Then, after a further 2 days, groups of six males were introduced to female vials and left for 2 hours to mate. Flies were unobserved during the mating period. However, a similar study using the same strain of *D. melanogaster* reported that >97% of females mated within a 2h window under a 3:2 male:female ratio (11). Females were then separated from males, transferred to the oviposition substrates and given 3-4 hr to lay eggs. Assays were all conducted under no choice conditions, with each treatment group exposed to one substrate type only. A sample size of 30 vials per treatment was used for each experiment. The number of eggs laid, and the number and size of egg clusters (defined as ≥2 eggs in physical contact) were recorded immediately following each assay, and the number of adult offspring were counted 12 days later. Further details of the oviposition substrates used for each of the three experiments are as follows:

(i) Effect of environmental microbes and nutrients on oviposition

To test the effect of environmental microbes (i.e. microbes occurring naturally in the environment, which colonise substrates in the absence of sterilization or preservatives) and the nutritional content of the oviposition substrate on egg placement, females were given one of the following oviposition substrates (n = 30 each): 1) standard SYA; 2) standard SYA lacking the preservatives propionic acid and Nipagin (methyl paraben); 3) low nutrient SYA, with 25% of the yeast and 25% of the sugar of standard SYA; and 4) low nutrient SYA lacking the preservatives propionic acid and Nipagin. Substrates lacking propionic acid and Nipagin showed visible microbial growth 48h after preparation. To prevent substrates being completely swamped with microbial colonies, all oviposition substrates were made only 24h prior to the assay. In addition to egg-to-adult development, we also scored the number of hatched eggs after 48 hours and the number of pupae after 7 days. Two vials were excluded from the hatching analysis because extensive microbial growth obscured hatching success. To

additionally test the effect of substrate condition (low nutrient SYA ± preservatives, n = 30 per treatment) on the extent of mixed-maternity egg clustering (≥ 1 egg in direct contact with ≥ 1 egg laid by ≥ 2 different females), we followed the same protocol but used a dye method to mark non-focal eggs. For this, non-focal females were reared from the larval stage, and maintained, on SYA diet containing 1400 ppm Sudan Black B (*Sigma-Aldrich #199664*) dissolved in corn oil (*Mazola*). Sudan Black B is an oil-soluble dye that binds lipids and thus becomes incorporated into the eggs of females feeding on the dye (33). Eggs laid by non-focal females appeared grey in colour, in contrast to the white of eggs laid by the non-dyed focals. We also used non-focal females with the *scarlet* eye marker mated to *scarlet* males, which allowed us to distinguish offspring of the focal females from those of the non-focals. Each female group consisted of 1 focal (wildtype, non-dyed) and 3 non-focal flies (*scarlet*, dyed).

Detailed statistical analysis: We analysed the effect of nutrient level and preservative presence on the total number of eggs laid using a two-part hurdle model from the *pscl* package (34). The probability of eggs being laid was modelled with a binomial distribution and logit link function, while a zero truncated negative binomial distribution with log link function was used for the count part of the model. Nutrient (2 levels: low, standard) and preservative (2 levels: absent, present) were fixed factors in both parts of the model. We analysed the effect of nutrient level and preservative presence on egg clustering proportion, egg hatchability, egg to pupa viability, egg to adult viability and hatched egg to adult viability using quasibinomial GLMs. The variable 'total eggs' was included as a fixed factor when modelling clustering proportion as a response variable, and 'clustering proportion' was included as a fixed factor when modelling egg hatchability as a response. There was significant collinearity between 'total eggs' and 'clustering proportion' as measured using a Pearson's correlation test from the *performance* package (35). Therefore, for all other measures of development, two separate models were run per response variable – one model included total eggs as a fixed factor and the other included clustering proportion. All models included nutrient and preservative as fixed factors. Reported significance values were derived using the Anova (Type II) function from the *car* package (36).

To test the accuracy of egg maternity scoring, we used the one-way intraclass correlation coefficient from the *irr* package (37) to test the agreement between focal (non-dyed) eggs counted and the number of focal (wildtype) offspring which eclosed from each vial. We also generated a Bland Altman Plot of focal eggs and focal offspring using the *BlandAltmanLeh* package (38).

(ii) Effect of antimicrobial preservatives alone on oviposition

To test the effect of antimicrobial preservatives on oviposition in the absence of microbes, we provided females with sterile oviposition substrates that contained or lacked individual preservatives. In contrast to the first experiment, above, here all substrates were sterile. Therefore, females were not exposed to environmental microbes at the start of the oviposition assay (regardless of whether preservatives were present). The oviposition substrates were as follows: (1) Standard SYA, (2) No preservatives (both Nipagin and propionic acid omitted), (3) Propionic acid only (Nipagin omitted), (4) Nipagin only (propionic acid omitted), (5) Ethanol only (Nipagin and propionic acid omitted). Where one or more preservatives were omitted, the equivalent volume of sterile RO water was added instead. Propionic acid and Nipagin both have general fungicidal and bactericidal properties, although Nipagin may be more effective against a greater diversity of microbes (39).

Detailed statistical analysis: We analysed the effect of antimicrobial preservatives on total eggs using a negative binomial GLM, and we analysed the effect of preservatives and total eggs on egg clustering proportion using a quasibinomial GLM. Preservatives (5 levels: standard; no preservatives; propionic acid only; Nipagin only; ethanol only) was included as a fixed factor in all models. Again, because of collinearity between total eggs and clustering proportion, when analysing the effect of preservatives on egg to adult viability we ran two separate quasibinomial GLMs. One model included preservatives and total eggs as fixed factors and the other included preservative and clustering proportion as fixed factors. All reported significance values were derived using the Anova (Type II) function from the *car* package.

(iii) Effect of commensal and pathogenic microbes on oviposition

In the third experiment, we tested the effect of fly-associated microbial communities on egg placement in the absence of preservatives. To do this, we used sterile oviposition substrates lacking preservatives, with or without microbial washes added to the surface of the substrate. Twenty-four hours before the oviposition assay, each oviposition substrate was spiked with 40 µl of one of the following washes: 1) negative control; 2) fly background control; 3) commensal microbes (i.e. members of the fly-associated microbiome); and 4) a culture of the bacteria *Alcaligenes faecalis* M3A. The species *A. faecalis* is an identified pathogen of *D. melanogaster*, exhibiting a 25% mortality rate upon larval ingestion in a previous study (26). The commensal microbe and fly background washes were made by placing 3 sterile grape juice agar plates into mini-cages with 300 adult flies per cage (1:1 sex ratio) for 10 hours (similar methodology used in (22)). The flies were then discarded, and each plate was repeatedly washed with 2.5 ml sterile RO H_2O. Half of this wash was used as the commensal microbe treatment, and the other half was filter sterilised to generate the fly background control (*Corning Costar* Spin-X centrifuge tube filter, 0.45 µm pore size, #8163). To generate the negative control wash, 2.5 ml RO H_2O was used to wash the surface of 3 separate sterile grape juice agar plates that remained unexposed to flies, and the entirety of this wash was filter sterilised to remove any microbial contaminants. Finally, an overnight culture of the gram-negative bacterium *A. faecalis* M3A was inoculated 1:100 into 100 ml Lysogeny Broth (5g NaCl, 10 g tryptone, 5 g yeast extract and 1.5 g glucose per litre H_2O) and grown at 30°C, 200 RPM for 3 hours, resulting in an optical density of 0.14 at 600 nm wavelength. 1 ml of this culture was centrifuged for 2 mins at 15,000 RPM and resuspended using 2 ml of the negative control wash to create the *A. faecalis* treatment. Following addition of the washes to the oviposition surfaces, vials were incubated for 24 hours at 25°C before the oviposition assay. To verify that the washes lead to differences in microbial environment, we checked the substrates 48 h following oviposition for visible microbial colonies. There were visible colonies in 90% of the commensal microbe substrates, compared with 27% of negative control substrates, 20% of fly background control substrates and 23% of *A. faecalis* substrates. A set of 5-6 unexposed vials from each treatment was incubated at 25°C for the duration of the experiment. These vials were spiked with the washes, but never exposed to flies, enabling us to

check the extent of microbial growth from the washes, separate to the microbes introduced by females during the oviposition assay. Of these unexposed substrates, there was visible microbial growth on 5 out of 6 commensal wash substrates, and 1 out of 5 negative control substrates, but no colonies were visible on any of the fly background control or *A. faecalis* substrates. Combined, these observations showed that, as intended, microbes were successfully transferred to the oviposition substrates in the commensal microbe wash, but not the negative or background controls.

Detailed statistical analysis: We analysed the effect of microbes on total eggs using a negative binomial GLM and analysed the effect of microbes and total eggs on egg clustering proportion using a quasibinomial GLM. Microbes (4 levels: negative control, fly background control, fly commensal microbes, *A. faecalis*) was a fixed factor in all models. As for the previous two experiments, we ran two separate quasibinomial GLMs for analysing effect of microbes on egg to adult viability. One model included microbes and total eggs as fixed factors, and the other included microbes and clustering proportion as fixed factors. All reported significance values were derived using the Anova (Type II) function from the *car* package.

Hypothesis 2 - *D. melanogaster* eggs exhibit broad spectrum antimicrobial activity.

Antimicrobial activity of egg surface molecules

To test if *D. melanogaster* eggs exhibit antimicrobial activity, we conducted radial diffusion assays according to (40). As a positive control, given Medfly eggs are known to exhibit antimicrobial activity (16), we also tested whole eggs and soluble material washed from the eggs of the Toliman Medfly strain, which was maintained as described in (41). The bacteria *Escherichia coli* dh5α, *Alcaligenes faecalis* M3A and *Micrococcus luteus* were grown overnight in Lysogeny Broth (LB, recipe as above) and the yeast *Saccharomyces cerevisiae* NYCC 505 was grown overnight in YPD medium (10 g yeast extract, 20 g peptone and 20 g glucose per litre H_2O). Overnight cultures were inoculated 1:100 into fresh broth and grown for 4 hours at 180 RPM and 30°C. 50 ml of each culture was centrifuged at 4°C for 10 mins, washed in 10 ml ice cold 10 mM sodium phosphate buffer, centrifuged once more and finally resuspended in 5 ml ice cold sodium phosphate buffer. The CFU/ml of the resuspended

microbial cultures was estimated from OD600 measurements and the volume equivalent to 2.54 x 10^6 CFU was used to inoculate the underlay agarose medium in the diffusion assay. The underlay medium for *E. coli*, *M. luteus* and *A. faecalis* consisted of 50 ml 100 mM sodium phosphate, 5 ml LB, 5 g agarose and 445 ml H2O. The underlay medium for *S. cerevisiae* was identical, but with 5 ml YPD broth in place of LB. Once autoclaved and cooled to 42°C, 7 ml of the underlay medium was mixed with ~2.54 x 10^6 CFU of the focal microbial species and poured into 90 mm petri dishes (*Fisher Scientific* #12654785) to make a very thin layer. Once set, a modified sterile p1000 Gilson pipette tip was used to punch four holes of ~2 mm in the underlay. One hole was punched for each of the samples being tested (two negative controls, *Drosophila* egg wash, and Medfly egg wash).

To generate the laid egg soluble material (LESM), we allowed *D. melanogaster* females to lay onto purple grape juice agar plates before picking 1000 eggs to 50 µl of PBS. For Medfly, eggs were collected dry as females pushed them through a mesh onto a piece of foil. 500 Medfly eggs (the equivalent approximate weight of 1000 *Drosophila* eggs) were transferred to 50 µl of PBS. Since purple grape juice agar plates contain Nipagen, a negative control for the *D. melanogaster* eggs was made by transferring a small piece of grape juice agar to 50 µl PBS, to control for any small amount of grape juice agar that may have been transferred with the eggs. After incubating the eggs or grape juice agar in PBS for 5 minutes, we centrifuged the samples to pellet the insoluble material and collected the supernatant. The supernatant (LESM) was passed through a spin filter before use in the assays (*Corning Costar* #8163).

We quantified the amount of protein in each LESM wash using a Qubit assay according to kit protocol, and then 2 µl of the LESM samples, or negative controls (PBS for Medfly eggs, PBS exposed to purple grape juice agar for *D. melanogaster* eggs) were applied to each of the wells of the underlay medium. The underlay was then incubated at 30°C for 3 hours. The overlay gel consisted of an enriched nutrient agarose. For *E. coli*, *M. luteus* and *A. faecalis* the overlay medium was made to the following recipe: 5 g NaCl, 10 g tryptone, 5 g yeast extract, 1.5 g glucose, 5 g agarose and 500 ml RO H_2O, and for *S. cerevisiae* the overlay medium was 10 g yeast extract, 20 g peptone, 20 g glucose, 5 g agarose and 500 ml H_2O. Once autoclaved and cooled to 42°C, 8 ml of overlay agarose was

poured over the underlay. Plates were then incubated at 30°C overnight. The following day, plates were photographed using a GXCAM HiChrome-S camera (*GT Vision Ltd.*) mounted on a Leica MZ75 dissecting microscope. The assay was then repeated using 0-30 intact eggs per well (rather than egg wash), against *E. coli*.

Results

Hypothesis 1 - *D. melanogaster* females plastically adjust egg placement based on the microbial environment.

(i) Effect of environmental microbes and nutrients on oviposition

We found that, independent of the nutritional content of the oviposition substrates, females laid fewer eggs when preservatives were absent (Figure 1A, Table S1-2, hurdle model with a negative binomial distribution, count part: $Z = 5.00$, $P < 0.0001$; binomial part: Z = 5.10, $P < 0.0001$). The most striking finding was that only 39.0% of female groups laid any eggs in the absence of preservatives, compared with 88.1% when preservatives were present. Nutrient level had no significant effect on the number of eggs laid (Figure 1A, Table S1-2). Although there were no microbial colonies visible on any substrate during the oviposition assay period, we noted that within 24 hours of the oviposition assay, microbial growth was visible on 81% of substrates lacking propionic acid and Nipagin (Figure S2, S4). Substrates containing preservatives did not exhibit visible microbial growth at any point. This observation suggests females were exposed only to actively growing microbial communities in vials lacking preservatives.

There was no significant effect of preservative presence or nutrient level on egg clustering proportion (Figure 1B, Table S3), but clustering proportion increased significantly with the total number of eggs ($F_{(1,67)} = 5.38$, P = 0.02; Figure S3, Table S4). Egg cluster sizes ranged from 2-9, with the largest clusters found on low nutrient substrates (Figure 1C). There were no significant effects of nutrient level, preservative presence or clustering proportion on egg hatching success, although hatching was lowest on standard, preservative-free substrates (Figure 1D, Table S5-6), which were also more quickly covered in mould-type growth than were low nutrient substrates (Figure S4). After 7 days, the proportion of laid eggs that had reached the pupal stage was significantly lower in the low nutrient treatments ($F_{(1,65)} = 131.94$, P < 0.0001), consistent with previous findings that lower nutrient

levels increase development time (e.g. (42)). There was no significant effect of preservative presence on pupariation. However, there was a significant effect of the interaction between nutrient level and clustering proportion on pupariation ($F_{(1,65)}$ = 5.07, P = 0.028) (Figure 1E, Table S9). There were no significant effects of nutrient level, preservatives, total eggs or clustering proportion on egg-to-adult viability or on hatched-egg-to-adult viability (Figure 1F-G, Tables S10-S15), and hatched-egg-to-adult viability was generally high (mean 94.8%, Fig 1G).

We tested the extent to which females clustered their eggs with those of other females and whether this was affected by substrate condition, by using dyed eggs and low nutrient media with and without preservatives. Across all vials, there were a grand total of 35 egg clusters containing ≥1 focal egg, and 25 of those clusters also contained ≥1 non-focal egg, meaning that focal eggs were part of a mixed maternity cluster in 71% of cases. Of the 35 clusters containing at least one focal egg, only 7 were found in the no-preservative treatment, and, of these 7, only 2 were of mixed maternity (additional details in Figure S6). There was significant agreement between the number of focal eggs scored and the number of focal offspring that eclosed from each vial (ICC = 0.96, $F_{(59,60)}$ = 55.1 , p = 1.39e-36, Figure S5), showing that focal and non-focal eggs were reliably distinguished.

(ii) Effect of antimicrobial preservatives alone on oviposition

To separate the confounding effects of microbe presence from the absence of preservatives, we tested for the effects of preservatives alone on oviposition, by using sterile substrates that contained or lacked different combinations of the standard antimicrobial preservatives used in SYA media. Overall, preservative treatment had a marginally significant effect on the number of eggs laid in each vial (Figure 2A, Tables S16-17). Compared with the standard treatment, which contained all preservatives, there were significantly fewer eggs laid when preservatives were completely absent, or when only Nipagin and/or ethanol were present (N + EtOH: Z = -2.53, P = 0.01; EtOH: Z = -2.01, P = 0.04; none: Z = -2.61, P = 0.009). There were also fewer eggs laid on substates that contained propionic acid, but lacked Nipagin and ethanol, when compared to the standard treatment, but this difference was not statistically significant (Z = -1.41, P = 0.16). There was no significant effect of preservative treatment on clustering proportion ($F_{(4, 144)}$ = 1.57, P = 0.19), but clustering proportion

significantly increased with the number of eggs laid ($F_{(1,144)}$, $P < 0.0001$, Figure S7, Table S19). Egg to adult viability was not significantly affected by treatment, total eggs or clustering proportion (Figure 2D, Tables S20-22).

(iii) Effect of commensal and pathogenic microbes on oviposition

To better control for the type of microbial environment experienced by ovipositing females, and to test whether commensal microbes elicit a different response to pathogenic microbes, we provided females with oviposition substrates containing fly-associated or pathogenic species. Despite established differences in microbial environment across vials (see Methods) there were no significant effects of treatment on the number of eggs laid, or the egg clustering proportion (Figure S8A-B, Table S23-26) although clustering proportion was again significantly affected by the total number of eggs ($F_{(1,110)} = 7.9$, $P = 0.006$, Figure S9, Table S26). Egg to adult viability was unaffected by substrate treatment and remained high at 88% despite extensive microbial growth in many vials (Figure S8D, Tables S27-29).

Hypothesis 2 - *D. melanogaster* eggs exhibit broad spectrum antimicrobial activity.

Antimicrobial activity of egg surface molecules

We found clear zones of growth inhibition around wells containing Medfly, but not *D. melanogaster* laid egg soluble material (LESM) for all four species of microbes tested (gram-negative strains *Escherichia coli* DH5α and *Alcaligenes faecalis* M3A, the yeast *Saccharomyces cerevisiae*, and the gram-positive bacteria *Micrococcus luteus)* (Figure 3). We quantified the total protein amount in the egg wash for each species using a Qubit protein assay. For the Medfly sample, the protein concentration was 834 µg/ml, equating to 83 ng per egg. For *D. melanogaster*, the protein concentration was below the limit of detection. Whole eggs from *D. melanogaster* also did not exhibit any antimicrobial activity when tested against *E. coli* (Figure S10).

Discussion

Our main aim was to test whether the microbial environment could be a driver of egg clustering behaviour in *D. melanogaster*. To do this, we conducted a series of experiments designed to untangle the effects of microbial community on oviposition from confounding factors, namely the presence or absence of antimicrobial preservatives. A novel aspect of our study was also to test for antimicrobial activity of *D. melanogaster* eggs, as has been found in other Dipteran species (16). Partially supporting our first hypothesis, we found that females adjusted the number of eggs they lay depending on the microbial environment present during oviposition, but that the extent of egg clustering was unaffected. Contrary to our second hypothesis, we found no evidence for broad-acting antimicrobial activity on *D. melanogaster* eggs. Overall, our results suggest the microbial environment is not a major driver of egg clustering behaviour in *D. melanogaster*.

The microbial environment affects number of eggs laid but not egg clustering (H1)

The most striking result of our first experiment was that most females refrained from egg laying completely when provided with oviposition substrates that lacked preservatives. This suggests that females were sensitive to the increased presence of actively growing environmental microbes (the consequence of leaving out preservatives). This was further supported by the results of the second experiment, in which we controlled for microbial environment by exposing females to sterilised substrates containing or lacking antimicrobial preservatives. Most females laid eggs on these sterilised substrates, regardless of the presence of preservatives, although they laid fewest eggs when propionic acid specifically was removed. Taken together, these results suggest that although the absence of propionic acid could partly explain reduced egg laying on substrates lacking preservatives in the first experiment, it was most likely the non-sterile environment that caused females to refrain from egg laying entirely.

Having established that non-sterile environments affect female oviposition, we then tested whether females responded differently to the type of microbial community present. We did not observe any differences in egg laying between females exposed to commensal (fly-associated)

microbes or the reportedly pathogenic bacterial species *A. faecalis* when compared with controls. Across all experiments, the extent of egg clustering was unaffected by microbial environment.

The relationship between the microbial environment and *D. melanogaster* oviposition is likely to be complex since microbes can have beneficial, neutral and/or negative impacts on flies, depending on microbial species and their abundances. *D. melanogaster* oviposit into microbe-rich decomposing fruit, and their larvae are dependent on beneficial yeasts for nutrition and normal development to adulthood. However, some bacteria and fungi (particularly moulds) are pathogenic when ingested by *D. melanogaster* larvae (26, 27). Therefore, females might refrain from ovipositing in non-sterile environments if they detect cues of pathogenic microbes. Indeed, detection of the microbial volatiles associated with pathogenic microbes (e.g. geosmin) leads to the suppression of feeding and egg-laying behaviours in *D. melanogaster* (43), a type of behavioural immunity (44). It is possible that instead of providing eggs with antimicrobials, and clustering to concentrate such defences, *D. melanogaster* simply avoid sites where they detect pathogens. Although we did not characterise the species of microbes growing on preservative-lacking substrates in the first experiment, we observed that most substrates harboured a mix of colony phenotypes, with several spore-bearing species characteristic of fungal moulds. Moulds such as *Penicillium* spp. are known to be detrimental to *D. melanogaster* development, likely due to the production of toxic secondary metabolites (43). It would be interesting to further characterise the identity and pathogenicity of the microbial species in vials in which females did and did not lay eggs. Most eggs laid developed to adulthood, suggesting that egg laying females made correct oviposition decisions, though we do not know whether non laying females were also 'correct'. This could be tested by manually adding eggs to rejected egg laying substrates and measuring their viability.

We tested responses of females to pathogenic microbes using the bacterial species *A. faecalis*, and found no effect of this microbe on oviposition. However, more extensive tests should be undertaken using different *A. faecalis* strains, doses and different pathogen species. A previous study of *A. faecalis* pathogenicity reported a 25% mortality rate upon larval ingestion (26), which was not seen in our experiment. It is possible that females did not alter their oviposition patterns because they

had insufficient cues of a pathogenic environment. No *A. faecalis* colonies were visible on the oviposition substrate, so it is possible the culture was not actively growing, or growing very slowly, which could reduce the probability of detection. Females also did not alter egg laying in response to commensal microbes. The diversity of commensal microbes (which should contain the transient gut microbiota of flies) is likely to be distinct from the environmental microbes that colonised the substrates in the absence of preservatives in the first experiment. There is evidence that *Drosophila* can distinguish between commensal and pathogenic microbes and select commensal-rich sites for egg-laying (24). The commensal microbial community can produce anti-fungal metabolites as well as provide access to nutrients which supports larval development (45, 46). These beneficial properties of a commensal microbial community could explain why females did not refrain from laying eggs in this assay.

Despite clear aversions to laying eggs in some microbial environments, we found no evidence that females altered egg clustering. Although females frequently laid eggs in clusters with those of conspecifics, mixed maternity clustering was not higher in microbial environments. Therefore, it seems unlikely that the microbial environment is a major driver of egg clustering, either in single or mixed maternity groups, at least under the conditions tested in this study. Although egg clustering is a widespread behaviour across taxa, the benefits of such behaviour remain largely unknown, though various protective functions have been suggested. For example, *Chlosyne lacinia* butterfly eggs have been shown to withstand desiccating conditions better when clustered, and eggs of the mite *Iphiseius degenerans* are better protected from predation when clustered within a domatium (9, 13). It is possible that *D. melanogaster* cluster eggs for similar reasons. *D. melanogaster* eggs are sensitive to desiccation in conditions below 80% relative humidity (47), so clustering eggs together in dry conditions may offer some protection. Although little is known about predation of *D. melanogaster* eggs in nature, ants have been shown to predate eggs in laboratory conditions, and flies appear to alter oviposition behaviour in response to hymenopteran cues (48). Egg clustering may either provide "safety in numbers" or minimise the chances of a predator encountering eggs. Females may also cluster eggs to take advantage of defence compounds other than antimicrobials. For example, *D.*

melanogaster coat their eggs with anticannibalism compounds (49). Another possibility is that laying in clusters is a social behaviour, which could lead to greater cooperation between hatching larvae. *Drosophila* larvae are able to coordinate their feeding movements to feed more effectively (11, 50) and larvae show greater aggregation on harder substrates, on which it is assumed feeding is more difficult (51). Indeed, although the overall proportion of eggs clustered was not affected by nutrient level, the largest clusters in our initial experiment were laid on low nutrient substrates. If egg clustering does lead to greater larval cooperation, this could be more important when nutrients are scarce. It remains to be investigated whether larvae emerging from clusters are better able to aggregate or coordinate feeding compared with larvae from eggs laid singly.

Egg clustering proportion did increase with the total number of eggs. This is consistent with a known pattern where grouped *D. melanogaster* females initially lay eggs in a dispersed way, with the extent of egg clustering increasing over time (11). A positive correlation between egg number and egg placement could indicate females are clustering by chance. However, this is unlikely for several reasons. First, *D. melanogaster* females do not lay batches of eggs in a rapid series, and appear to evaluate each individual oviposition site location before depositing each egg (52). Second, the substrate surface areas used in our experiments were not limiting in space - similar numbers of eggs could have been laid in a completely dispersed manner. Third, comparisons of real egg clustering data with null models simulating random placement support the idea that females distribute their eggs in non-random patterns across individual substrates (11).

Drosophila melanogaster eggs are not provisioned with broad-spectrum antimicrobial compounds (H2)

If *D. melanogaster* eggs are provisioned with diffusible antimicrobials, as seen in some other Dipteran species (16), females might cluster eggs more in microbially diverse environments in order to increase the concentration of protective antimicrobial compounds either from their own eggs or from those of other females. However, counter to our hypothesis, there was no evidence that *D. melanogaster* females provisioned the surfaces of their eggs with soluble, broad-spectrum antimicrobial peptides, as

occurs in Medfly (16). Since *D. melanogaster* oviposit in microbially-rich environments and are reliant on microbial phytophagy to break down fruits and provide nutrients, broad-spectrum antimicrobials on egg surfaces could be detrimental for this species if they deplete beneficial microbial species.

Despite the evidence that AMP genes encoding antimicrobial peptides are expressed and enriched for expression in the female reproductive tract, none of the 21 known AMPs, or the 12 Bomanin peptides (53) were found among the 1840 proteins identified in a recent proteomic study of the female reproductive tissue and luminal fluid (54). Regardless, the absence of antimicrobial activity in our diffusion assays suggests *D. melanogaster* do not provision their eggs with antimicrobial defences that could be exploited as public goods. Instead, it is more likely that *D. melanogaster* protect their offspring from infection by avoiding ovipositing into sites containing pathogens (behavioural immunity (44)) or choosing sites where the microbial community itself is producing antimicrobials against entomopathogenic species (45).

Propionic acid as an oviposition cue

One surprising finding of our study was that propionic acid was a positive fecundity cue. We found that under sterile conditions, females laid fewest eggs on substrates that lacked propionic acid. Although antimicrobial preservatives such as propionic acid are added to artificial diets to control growth of mould and bacteria, they may also resemble microbial-derived metabolites that act as positive cues in natural oviposition sites. For example, yeast and bacteria produce short-chain fatty acids (SCFA) including propionic acid during fruit decomposition, and *Drosophila* possess neurons that are specifically activated by such acids (55). While adult flies have a *positional* aversion to higher concentrations of propionic acid (2.5%, vs 0.3% used in the current study) (56), this seems to be uncoupled from visiting a site for oviposition, and female *D. melanogaster* exhibit attraction towards oviposition substrates containing some SCFAs (57). *D. melanogaster* larvae are also attracted to propionic acid, and supplementation of nutrient-poor media with 1% propionic acid can improve larval survival (56). It is therefore possible that females increase egg laying at specific levels of

propionic acid as this represents a beneficial developmental environment for their offspring. Similarly, ethanol (used to solubilise Nipagin) is one of the main metabolites of fermentation. Female *D. melanogaster* prefer to oviposit in ethanol-supplemented medium (58), although neither Nipagin or ethanol were observed to have any significant effect on the number of eggs laid in our study.

Conclusions

In this study we have shown that females are sensitive to the microbial environment and laid more eggs in a sterile environment. However, we found that *D. melanogaster* eggs do not exhibit antimicrobial activity, and that egg clustering was unchanged across the environments tested. These findings suggest *D. melanogaster* females do not cluster their eggs to gain public goods benefits from the communal production of antimicrobial compounds. Therefore, future studies should focus on finding alternative explanations for egg clustering behaviour.

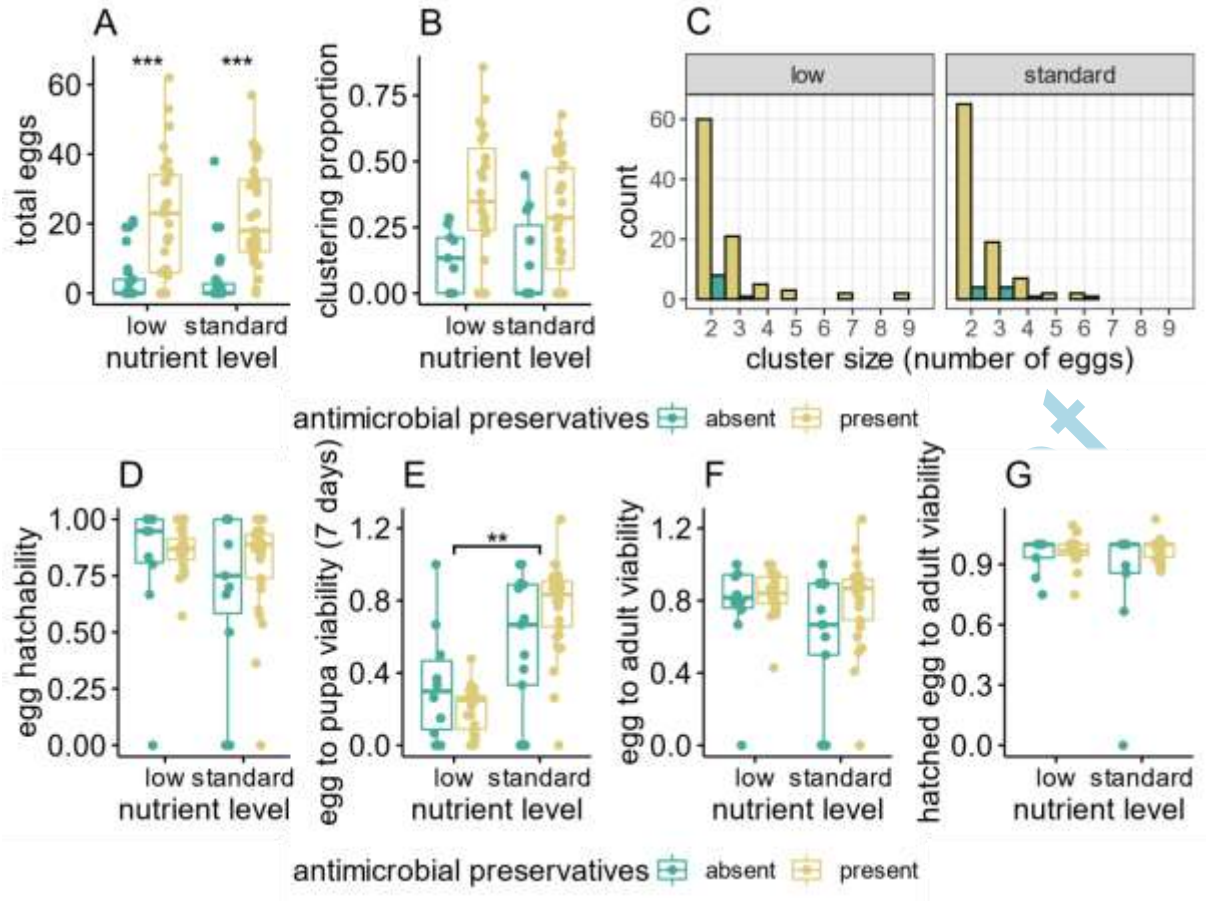


Figure 1. Females lay fewer eggs when antimicrobial preservatives are absent from the substrate. Oviposition substrates had low or standard levels of nutrients (yeast and sugar), and antimicrobial preservatives were either absent (green boxes and bars) or present (yellow boxes and bars). (A) total eggs laid by 4 females per oviposition vial. (B) the proportion of total eggs in each vial that were clustered (total clustered eggs / total eggs). (C) the frequency of egg cluster sizes seen in each substrate treatment. Data are combined from across all vials in each treatment. (D) the proportion of total eggs hatched after 48h (number of hatched eggs / total eggs) in each vial. (E) the proportion of total eggs developed into pupae after 7 days (number of pupae / total eggs) in each vial. (F) the proportion of total eggs that developed into adults (total adult offspring / total eggs) in each vial. (G) the proportion of hatched eggs that developed into adults (total adult offspring / number of hatched eggs) in each vial. Boxplots show the interquartile range (IQR) and median, and whiskers represent the largest and smallest values within 1.5 times the IQR above and below the 75th and 25th percentiles, respectively. Raw data points are plotted with jitter. Statistically significant differences between treatments are indicated, using p values estimated from model testing (*** $p < 0.0001$, ** $p < 0.001$).

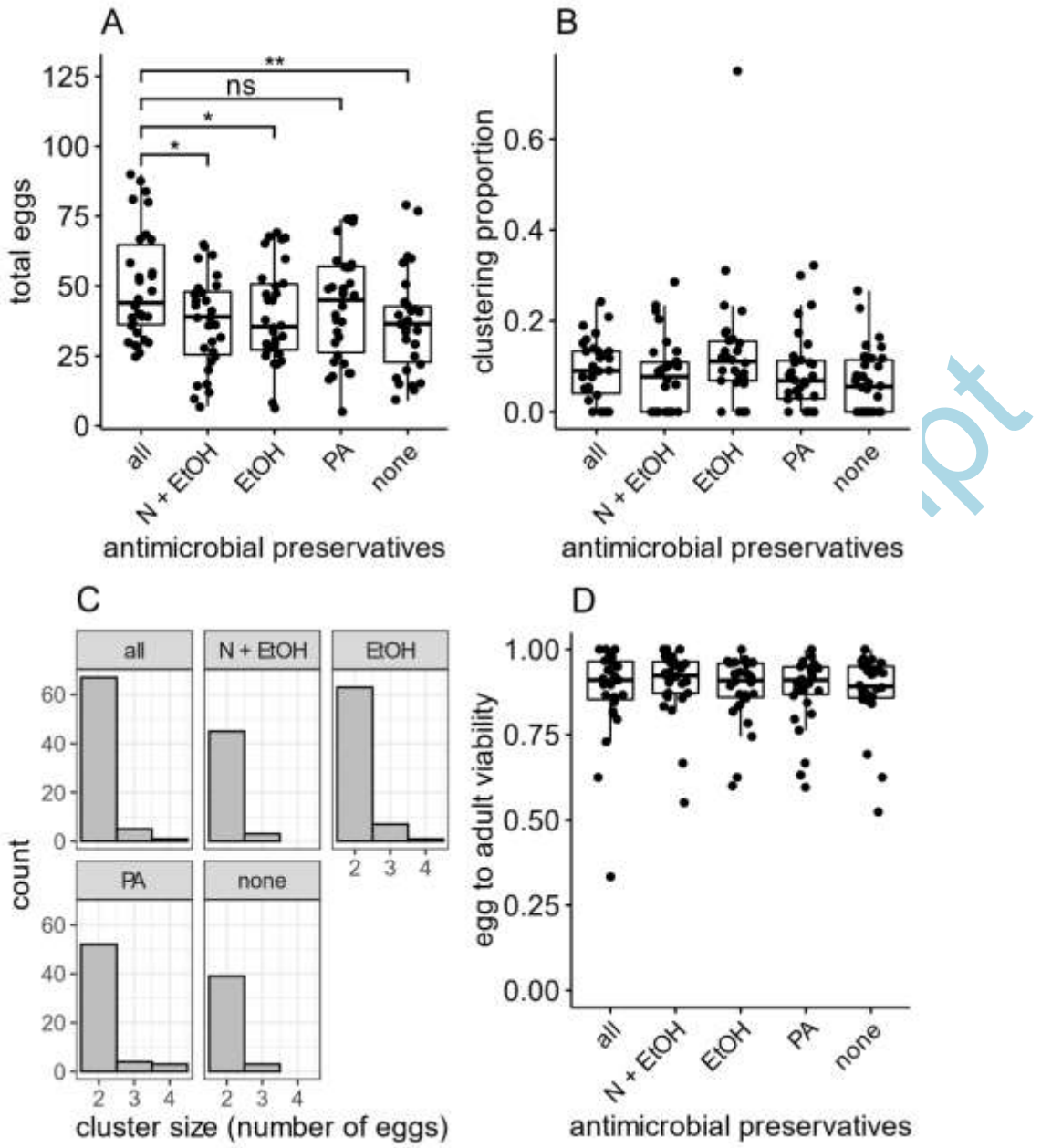


Figure 2. Antimicrobial preservatives have a marginally significant effect on fecundity. Oviposition substrates contained Nipagin and propionic acid ("all"), Nipagin ("N + EtOH"), Ethanol only ("EtOH"), propionic acid only ("PA") or no preservatives ("none"). (A) total eggs laid by 4 females per oviposition vial. (B) the proportion of total eggs in each vial that were clustered (total clustered eggs / total eggs). (C) the frequency of egg cluster sizes seen in each substrate treatment. Data are combined from across all vials in each treatment. (D) the proportion of total eggs that developed into adults (total adult offspring / total eggs) in each vial. Boxplots are as decribed for Figure 1. Statistical significance indicated in (A) (* $p < 0.01$; ** $p < 0.001$; ns: $p > 0.05$) with p values derived from model summary.

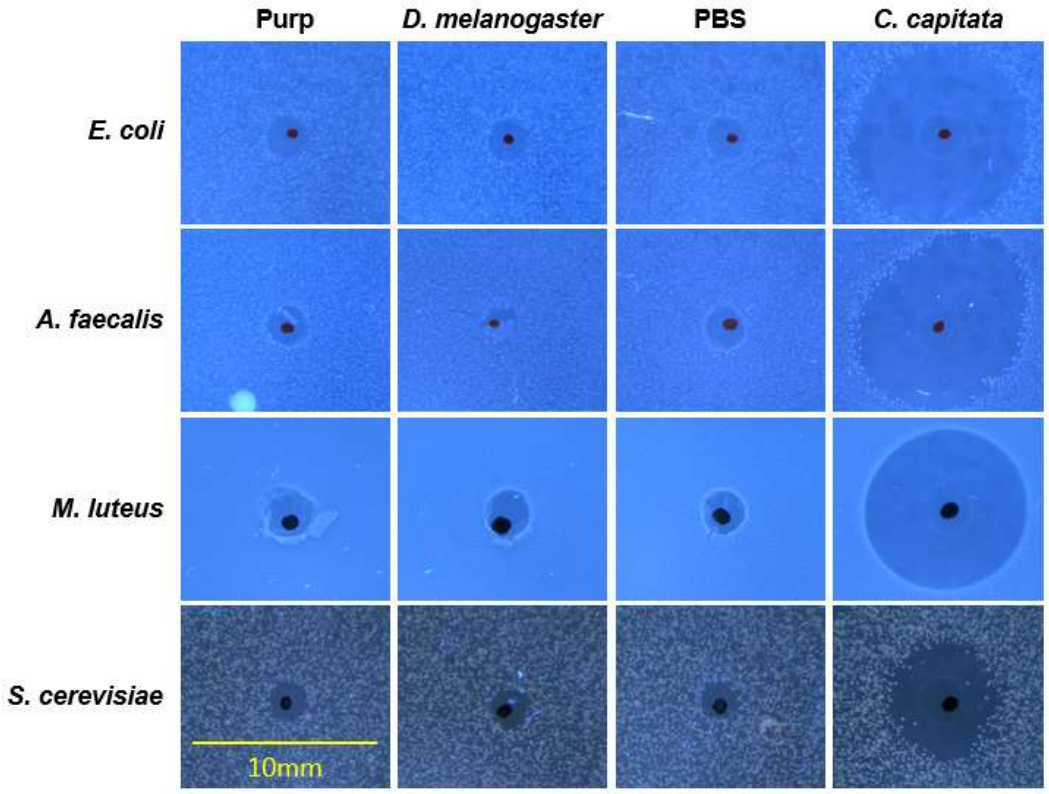


Figure 3. Laid egg soluble material of *Drosophila melanogaster* does not exhibit broad-spectrum antimicrobial activity. Soluble material from washing freshly laid *D. melanogaster* or *C. capitata* eggs was pipetted directly into wells in the assay plates. The negative control for *D. melanogaster* was PBS that had been washed over purple grape juice agar (column 1, "Purp"), and the negative control for *C. capitata* was PBS only (column 3). Each plate contained a live culture of either *Escherichia coli* dh5α, *Alcaligenes faecalis* M3A, *Micrococcus luteus* or *Saccharomyces cerevisiae* NYCC 505. Individual wells were photographed under a microscope to show any zones of growth inhibition surrounding the well. The centre of each well was marked with a black dot on the petri dish for ease of identification. A 10 mm scale bar is shown at the bottom left.

References

1. Resetarits Jr, WJ. 1996. Oviposition site choice and life history evolution. *Amer. Zool*. **36**, 205-15.

2. Refsnider JM, Janzen FJ. 2010. Putting eggs in one basket: ecological and evolutionary hypotheses for variation in oviposition-site choice. *Annu. Rev. Ecol. Evol. Syst.* **41**, 39-57.

3. Rudolf VHW, Rödel M-O. 2005. Oviposition site selection in a complex and variable environment: the role of habitat quality and conspecific cues. *Oecologia*. **142**, 316-25.

4. Björkman C, Larsson S, Bommarco R. 1997. Oviposition preferences in pine sawflies: a trade-off between larval growth and defence against natural enemies. *Oikos*. **79**, 45-52.

5. Deas JB, Hunter MS. 2013. Delay, avoidance and protection in oviposition behaviour in response to fine-scale variation in egg parasitism risk. Anim. Behav. **86**:933-40.

6. Doody JS, Freedberg S, Keogh JS. 2009. Communal egg- laying in reptiles and amphibians: evolutionary patterns and hypotheses. *Q. Rev. Biol.* **84**, 229-52.

7. Riehl C. Evolutionary routes to non-kin cooperative breeding in birds. Proceedings Biological sciences. 2013;280(1772):20132245.

8. Welsh DP, Fuller RC. 2011. Where to place your eggs: the effects of conspecific eggs and water depth on oviposition decisions in bluefin killifish. *J. Zool.* **284**, 192-7.

9. Faraji F, Janssen A, Sabelis MW. 2002. The benefits of clustering eggs: the role of egg predation and larval cannibalism in a predatory mite. *Oecologia.* **131**, 20-6.

10. Courtney SP. 1984. The evolution of egg clustering by butterflies and other insects. *Am. Nat.* **123**, 276-81.

11. Churchill ER, Fowler EK, Friend LA, Archetti M, Yu DW, Bourke AFG, Chapman T, Bretman A. 2024. Female fruit flies use social cues to make egg clustering decisions. *BioRxiv.* Preprint article.

12. Janz N. 2003. Evolutionary ecology of oviposition strategies. In: Chemoecology of insect eggs and egg deposition, edited by Hilker M & Meiners T. Blackwell Publishing, Berlin (Germany). 349-76.

13. Clark BR, Faeth SH. 1998. The evolution of egg clustering in butterflies: A test of the egg desiccation hypothesis. *Evol. Ecol.* **12**, 543-52.

14. Hilker M, Blum MS. 2003. Chemical protection of insect eggs. In: Chemoecology of insect eggs and egg deposition, edited by Hilker M & Meiners T. Blackwell Publishing, Berlin (Germany). 61-83.

15. Levin SA. 2014. Public goods in relation to competition, cooperation, and spite. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 10838-45.

16. Marchini D, Marri L, Rosetto M, Manetti AG, Dallai R. 1997. Presence of antibacterial peptides on the laid egg chorion of the Medfly *Ceratitis capitata*. *Biochem. Biophys. Res. Commun.* **240**, 657-63.

17. Rosetto M, Marchini D, de Filippis T, Ciolfi S, Frati F, Quilici S, Dallai R. 2003. The ceratotoxin gene family in the Medfly *Ceratitis capitata* and the Natal fruit fly *Ceratitis rosa* (Diptera: Tephritidae). *Heredity*. **90**, 382-9.

18. Ferrandon D, Jung AC, Criqui M, Lemaitre B, Uttenweiler- Joseph S, Michaut L, Reichhart J, Hoffmann JA. 1998. A drosomycin–GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J*. **17**, 1217-27.

19. Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart J-M, Lemaitre B, Hoffmann JA, Imler JL. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity*. **13**, 737-48.

20. Charlet M, Lagueux M, Reichhart JM, Hoffmann D, Braun A, Meister M. 1996. Cloning of the gene encoding the antibacterial peptide drosocin involved in *Drosophila* immunity: expression studies during the immune response. *Eur. J. Biochem*. **241**, 699-706.

21. McDonough-Goldstein CE, Borziak K, Pitnick S, Dorus S. 2021. *Drosophila* female reproductive tract gene expression reveals coordinated mating responses and rapidly evolving tissue-specific genes. *G3*. **11**.

22. Sato A, Tanaka KM, Yew JY, Takahashi A. 2021. *Drosophila suzukii* avoidance of microbes in oviposition choice. *R. Soc. Open Sci.* **8**, 201601.

23. Li H, Ren L, Xie M, Gao Y, He M, Hassan B, Lu Y, Cheng D. 2020. Egg-surface bacteria are indirectly associated with oviposition aversion in *Bactrocera dorsalis*. *Curr. Biol.* **30**, 4432-40.

24. Liu W, Zhang K, Li Y, Su W, Hu K, Jin S. 2017. Enterococci mediate the oviposition preference of *Drosophila melanogaster* through sucrose catabolism. *Sci. Rep*. **7**, 13420.

25. Shin SC, Kim S-H, You H, Kim B, Kim AC, Lee K-A, Yoon J-H, Ryu J-H, Lee W-J. 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*. **334**, 670-4.

26. Bing XL, Winkler J, Gerlach J, Loeb G, Buchon N. 2021. Identification of natural pathogens from wild *Drosophila suzukii*. *Pest Manag. Sci.* **77**, 1594-606.

27. Keebaugh ES, Schlenke TA. 2014. Insights from natural host–parasite interactions: The *Drosophila* model. *Dev. Comp. Immunol.* **42**, 111-23.

28. R Core Team. 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

29. Wickham H. 2016. ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag.

30. Kassambara A. 2020. ggpubr: 'ggplot2' Based Publication Ready Plots.

31. Hope RM. 2022. Rmisc: Ryan Miscellaneous.

32. Hartig F. 2022. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.

33. Vilarinho EC, Fernandes OA, Omoto C, Hunt TE. 2006. Oil-Soluble Dyes for Marking *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **99**:2110-5.

34. Jackman S. 2020. pscl: Classes and methods for R developed in the political science computational laboratory.

35. Lüdecke D, Ben-Shachar MS, Patil I, Waggoner P, Makowski D. 2021. performance: An R package for assessment, comparison and testing of statistical models. *J. Open Source Softw.* **6**.

36. Fox J, Weisberg S. 2018. An R companion to applied regression. Sage publications.

37. Gamer M, Lemon J, Fellows I, Singh P. 2019. irr: Various Coefficients of Interrater Reliability and Agreement.

38. Lehnert B. 2015. BlandAltmanLeh: Plots (Slightly Extended) Bland-Altman Plots.

39. Téfit MA, Gillet B, Joncour P, Hughes S, Leulier F. 2018. Stable association of a *Drosophila*-derived microbiota with its animal partner and the nutritional environment throughout a fly population's life cycle. J. Insect Physiol. **106**:2-12.

40. Steinberg DA, Lehrer RI. 1997. Designer assays for antimicrobial peptides. In: Antibacterial Peptide Protocols, edited by Shafer WM. Humana Press (Totowa, NJ). **169-86**.

41. Darrington M, Leftwich PT, Holmes NA, Friend LA, Clarke NVE, Worsley SF, Margaritopolous JT, Hogenhout SA, Hutchings MI, Chapman T. 2022. Characterisation of the symbionts in the Mediterranean fruit fly gut. *Microb. Genom.* **8**, 000801.

42. Duxbury EML, Chapman T. 2019. Sex-specific responses of life span and fitness to variation in developmental versus adult diets in *Drosophila melanogaster*. *J. Gerontol. A. Biol. Sci. Med. Sci.* **75**, 1431-8.

43. Stensmyr MC, Dweck HKM, Farhan A, Ibba I, Strutz A, Mukunda L, Linz J, Grabe V, Steck K, Lavista-Llanos S, Wicher D, Sachse S, Knaden M, Becher PG, Seki Y, Hansson BS. 2012. A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell*. **151**, 1345-57.

44. De Roode JC, Lefèvre T. 2012. Behavioral Immunity in Insects. *Insects*. **3**,789-820.

45. Fischer CN, Trautman EP, Crawford JM, Stabb EV, Handelsman J, Broderick NA. 2017. Metabolite exchange between microbiome members produces compounds that influence *Drosophila* behavior. *eLife*. **6**, e18855.

46. Grenier T, Leulier F. 2020. How commensal microbes shape the physiology of *Drosophila melanogaster*. *Curr. Opin. Insect Sci*. **41**, 92-9.

47. Al-Saffar ZY, Grainger JNR, Aldrich J. 1995. Influence of constant and changing temperature and humidity on the development and survival of the eggs and pupae of *Drosophila melanogaster* (Meigen). *J. Therm. Biol.* **20**:389-97.

48. Davis SM, Chism GT, Maurer MM, Trejo JE, Garcia RJ, Schlenke TA. 2021. A hymenopteran odorant alerts flies to bury eggs. *BioRxiv*. 462443.

49. Narasimha S, Nagornov KO, Menin L, Mucciolo A, Rohwedder A, Humbel BM, Stevens M, Thum AS, Tsybin YO, Vijendravarma RK. 2019. *Drosophila melanogaster* cloak their eggs with pheromones, which prevents cannibalism. *PLoS Biology*. **17**:e2006012.

50. Dombrovski M, Poussard L, Moalem K, Kmecova L, Hogan N, Schott E, Vaccari A, Acton S, Condron B. 2017. Cooperative behavior emerges among *Drosophila* larvae. *Curr. Biol.* **27**, 2821-6.

51. Durisko Z, Kemp R, Mubasher R, Dukas R. 2014. Dynamics of social behavior in fruit fly larvae. *PLoS One.* **9**, e95495.

52. Yang C-H, Belawat P, Hafen E, Jan LY, Jan Y-N. 2008. Drosophila egg-laying site selection as a system to study simple decision-making processes. *Science*. **319**, 1679-83.

53. Hanson MA, Lemaitre B. 2020. New insights on *Drosophila* antimicrobial peptide function in host defense and beyond. *Curr. Opin. Immunol*. **62**, 22-30.

54. McDonough-Goldstein CE, Whittington E, McCullough EL, Buel SM, Erdman S, Pitnick S, Dorus, S. 2021. Pronounced postmating response in the *Drosophila* female reproductive tract fluid proteome. *Mol Cell Proteomics*. **20**, 100156.

55. Ai M, Min S, Grosjean Y, Leblanc C, Bell R, Benton R, et al. Acid sensing by the Drosophila olfactory system. Nature. 2010;468(7324):691-5.

56. Depetris-Chauvin A, Galagovsky D, Chevalier C, Maniere G, Grosjean Y. 2017. Olfactory detection of a bacterial short-chain fatty acid acts as an orexigenic signal in *Drosophila melanogaster* larvae. *Sci. Rep*. **7**, 14230.

57. Joseph RM, Devineni AV, King IF, Heberlein U. 2009. Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 11352-7.

58. Azanchi R, Kaun KR, Heberlein U. 2013. Competing dopamine neurons drive oviposition choice for ethanol in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 21153-8.