BIOLOGY LETTERS

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Research

Cite this article: Liu X, Brisson JA. 2023 Dopamine mediates the pea aphid wing plasticity. *Biol. Lett.* **19**: 20230024. https://doi.org/10.1098/rsbl.2023.0024

Received: 25 February 2023
Accepted: 26 April 2023

Subject Areas:
ecology, evolution, developmental biology

Keywords:
dopamine, phenotypic plasticity, wing dimorphism, pea aphid

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6631839.

THE ROYAL SOCIETY PUBLISHING

Evolutionary developmental biology

Dopamine mediates the pea aphid wing plasticity

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Many organisms exhibit phenotypic plasticity, in which developmental processes result in different phenotypes depending on their environmental context. Here we focus on the molecular mechanisms underlying that environmental response. Pea aphids (*Acyrthosiphon pisum*) exhibit a wing dimorphism, in which pea aphid mothers produce winged or wingless daughters when exposed to a crowded or low-density environment, respectively. We investigated the role of dopamine in mediating this wing plasticity, motivated by a previous study that found higher dopamine titres in wingless- versus winged-producing aphid mothers. In this study, we found that manipulating dopamine levels in aphid mothers affected the numbers of winged offspring they produced. Specifically, asexual female adults injected with a dopamine agonist produced a lower percentage of winged offspring, while asexual females injected with a dopamine antagonist produced a higher percentage of winged offspring, matching expectations based on the titre difference. We also found that genes involved in dopamine synthesis, degradation and signalling were not differentially expressed between wingless- and winged-producing aphids. This result indicates that titre regulation possibly happens in a non-transcriptional manner or that sampling of additional timepoints or tissues is necessary. Overall, our work emphasizes that dopamine is an important component of how organisms process information about their environments.

1. Introduction

Adaptive phenotypic plasticity describes the evolved ability of an organism to produce a range of phenotypes in response to environmental cues [1]. One important frontier in plasticity research is investigating how organisms process and convert environmental information into molecular responses, giving rise to alternative, adult phenotypes [2]. Investigating this question might provide key insights into how environmental and genetic signals are integrated during development, and how the system is free or constrained to evolve in the future.

Here we use the wing plasticity of the pea aphid, *Acyrthosiphon pisum*, as a model for studying the molecular mechanisms underlying phenotypic plasticity. Adult females can be winged or wingless, with the two morphs being a classic example of an evolutionary trade-off between dispersal and reproduction, respectively [3]. During the asexual part of the pea aphid's life cycle, females exclusively produce genetically identical daughters parthenogenetically via live birth of nymphs (not via oviparous egg production). More crowded conditions cause aphid mothers to produce an increased proportion of winged daughters, while lower-density conditions result in mainly wingless daughters [4]. Because it is the mother that perceives the cue and the daughters that respond, this is a transgenerational plasticity.

We focus our attention on dopamine signalling as a candidate molecular signalling pathway for mediating the aphid wing plasticity. Dopamine is a type of catecholamine neurotransmitter present throughout Animalia that is

involved in a range of important, environmentally responsive phenotypes [5–7]. We previously showed that dopamine titre levels were higher in pea aphid mothers under solitary, wingless-daughter producing conditions compared to pea aphid mothers under crowded, winged-daughter producing conditions [8], suggesting that the dopamine signalling pathway might be involved in the aphid wing plasticity. Here we sought to determine if dopamine has a causal role in this plasticity by manipulating dopamine levels via pharmaceutical injections. We hypothesized that injecting a dopamine agonist would decrease the percentage of winged offspring produced by injected mothers, while injecting a dopamine antagonist would increase the percentage of winged offspring. We also examined gene expression levels of dopamine pathway-related genes in crowded versus solitary conditions. Our results support a role for dopamine in the pea aphid wing plasticity and contribute to a wider literature on the role of dopamine signalling in phenotypic plasticity.

2. Material and methods

(a) Aphid stocks

Two lines, ROC1 and SSC3, were used for this study. We used two different lines to test the generality of our results, i.e. to make sure our results were not line-specific. Stocks were reared on *Vicia faba* seedlings (Improved Long Pod, Harris Seeds) in an incubator with long-day conditions (16 L : 8 D), relative humidity of 30 ± 10%, and temperature of 19 ± 3.5°C. Under these conditions, aphids reproduce asexually and viviparously. Each seedling was grown in a single plastic plant pot (S17648, Fisher Scientific) with a cylindrical cage cut from clear PETG tubes (VISIPAK) sealed with a net (Noseeum netting, 117 inches, Barre Army Navy Store) to retain aphids. Before each experiment, aphids were reared at four to six individuals per cage for at least two generations to remove any density effects across generations.

(b) Pharmaceutical injections

A dopamine agonist (apomorphine, Sigma) and antagonist (flupenthixol, Fisher Scientific) were used for injections. Pea aphids of genotype SSC3 were maintained at six per cage for at least two generations before agonist injection and four per cage before antagonist injection. The rearing density was lower for the latter in order to minimize the production of winged aphids. ROC1 aphids were reared at four per cage for both types of injection because ROC1 aphids produce more winged offspring at a density of six per cage than SSC3.

We chose these rearing densities with the goal of producing an intermediate amount of winged offspring with each experiment, based on our previous experience with these two lines. Our goal was an intermediate offspring percentage because we hypothesized that agonist injection would decrease the winged percentage and the antagonist injection would do the opposite. It is important to note that the wing plasticity response cannot be controlled precisely and thus varies from experiment to experiment. Thus, the relevant comparisons in our study are always *within* experiments between treatment and control samples.

Healthy adult females that were 2–3 days after their final moult were pooled and randomly divided into experimental or control injection groups. Pea aphids of this stage are actively producing offspring. Aphids were injected with 1 µg µl^{-1} apomorphine (Sigma) or 1 µg µl^{-1} flupenthixol (Fisher Scientific) using a pulled glass capillary needle in separate experiments. Both apomorphine and flupenthixol were dissolved in Ringer's solution. Control insects in each experiment were injected with Ringer's. Glass needles were prepared using a micropipette puller (P-1000, Sutter Instrument) with the setting of pull = 150 and heat = 504. Needle tips were broken against a glass slide edge. Injection was done with a microinjector (PLI-10, Warner Instruments) for 0.1 s, 5.7 psi, injecting 0.3 µl of liquid. Injection concentrations were determined after considering experiments with similar agonists and antagonists used at the higher concentrations of 10 µg µl^{-1} to 80 µg µl^{-1} in locusts, which are larger insects [9]. After injection, aphid mothers were transferred onto caged plants with a density of three mothers per cage for 24 h for reproduction. Injected adult females were then removed while their offspring were kept growing on the plants. Offspring were categorized as winged or wingless after reaching at least the third instar stage, when wing buds become visible. For each cage, the offspring's winged morph percentage was calculated as the number of winged offspring divided by the total number of offspring. We counted each cage as a biological replicate. Because extremely low numbers of offspring could lead to misleading winged morph percentage values, we excluded cages with low numbers of progeny (less than six) from analyses. The original and filtered replicate numbers for each experiment are shown in electronic supplementary material, table S1.

(c) Expression level analysis of dopamine-related genes

We examined expression levels of 21 genes involved in dopamine synthesis, degradation and signalling (D1-like receptor, D2-like receptor, DAT1, DAT2, DAT3, DAT4, DopEcR, VMAT, Prt, henna, ple, Ddc, Tan, ebony, MAO, DBH, AD/ALDH, Black, Laccase, aaNAT, Y-y, electronic supplementary material, table S2). Five previously generated RNA-seq datasets ([8,10] and unpublished data) were used to gather gene expression level information. The samples for these RNA-Seq datasets were adult asexual female bodies, with ovaries and embryos removed, of five unique genetic lines: 319, C74, 200, 218 and ROC1. All RNA-Seq datasets compared crowded (winged offspring-producing) and solitary (wingless offspring-producing) aphids. Sample preparation and treatment details for 319 and C74 can be found in reference [10]. Sample collection for lines 200 and 218 (unpublished) followed the same protocol as [10]. For these four lines (319, C74, 200, 218), there were three biological replicates per treatment (crowded versus solitary) and the crowding or solitary treatment lasted 12 h. Each replicate comprised eight dissected adult carcasses. Sample preparation details for ROC1 can be found in reference [8]. For this line, there were four biological replicates for each treatment and the treatment lasted 16 h. Fifteen dissected adult carcasses were used for each replicate. The GenBank accession numbers for line 319 are SRR13238533-SRR13238536, SRR13238541 and SRR13238542; for line C74 are SRR1323853-SRR13238540, SRR13238543 and SRR13238544; for line ROC1 are SRR2148902-SRR2148909; for line 200 are SRR21747212-SRR21747205; for line 218 are SRR21747204-SRR21747209.

We processed raw reads using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We trimmed adaptor sequences, filtered out poor-quality sequences (quality score cut-off of 20), and discarded sequences shorter than 20 nt. The resulting reads were then mapped to the pea aphid genome v3.0 (https://bipaa.genouest.org/sp/acyrthosiphon_pisum/download/genome/v3.0/) using HISAT2 [11]. Gene records with no mapped reads were removed. Count normalization and differential expression analysis was done with R package DESeq2 using default settings [12], such that the *p*-values generated by a Wald test were corrected via the Benjamini and Hochberg method [13].

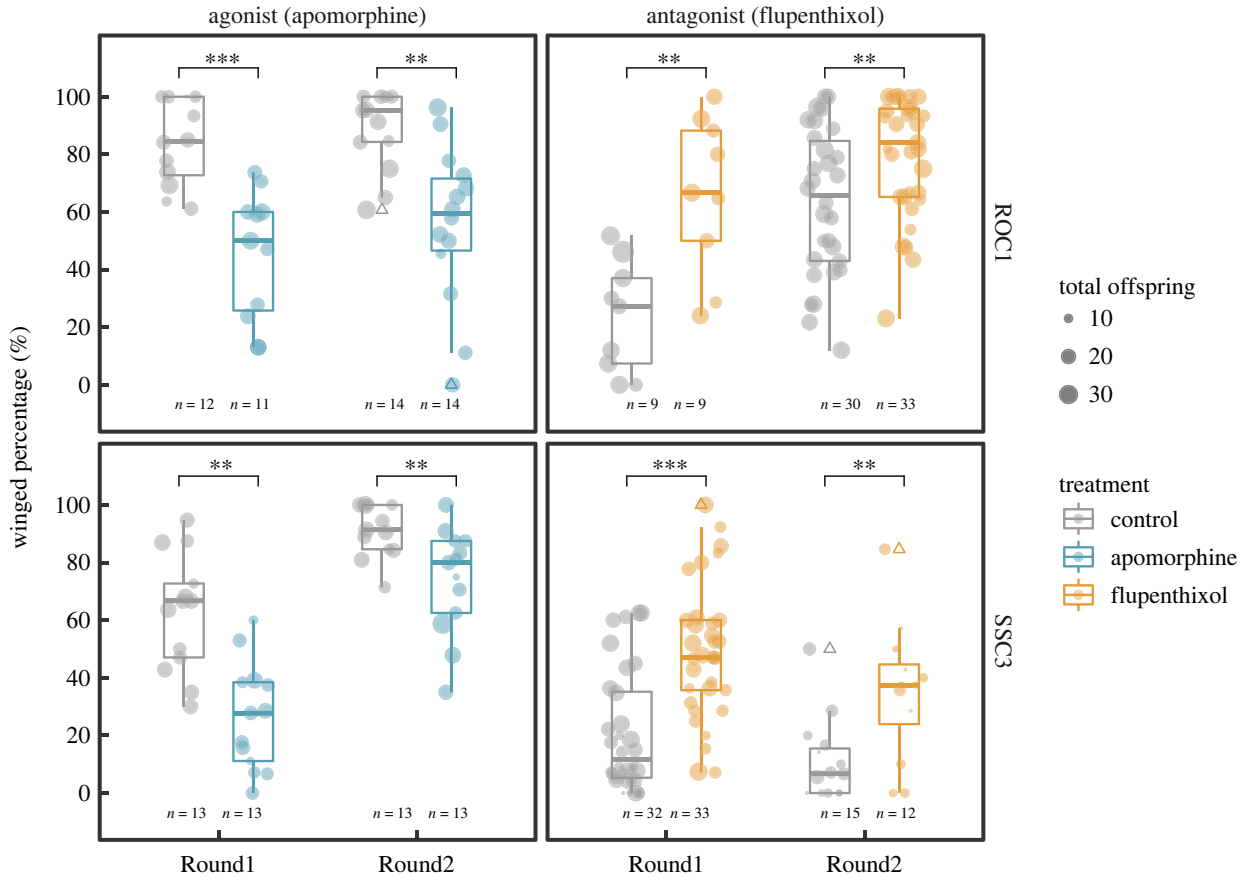

Figure 1. Manipulating dopamine levels in pea aphid mothers changes the percentage of winged daughters they produce. The *y*-axis shows the winged percentage of offspring from injected aphid mothers. Breaks on the *x*-axis show two different experiment rounds (1 and 2). Each data point represents one biological replicate (comprising all the offspring from three aphid mothers produced within the first 24 h after injection; we only retained replicates with six or more offspring), with the total number of biological replicates shown for each treatment (*n*). The size of the point gives a relative indication of the number of offspring that contributed to that replicate. Triangles represent statistical outliers for the boxplot. Asterisks show significance level (Wilcoxon rank-sum tests; *adjusted *p*-value ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

(d) Statistics

Statistics were conducted using R 3.6.3. Normality was assessed using Shapiro–Wilk normality tests. Wing percentage comparisons were analysed using Wilcoxon rank-sum tests, as the data were not normally distributed. Total aphid numbers per cage comparisons were analysed using a *t*-test, as those data were normally distributed. *p*-Values were adjusted using the Benjamini & Hochberg correction [13].

3. Results

We used pharmaceutical injections to examine a potential role of dopamine signalling in the pea aphid wing plasticity. To mimic increases in dopamine levels, we used apomorphine, a non-selective dopamine agonist that works on Type I and Type II dopamine receptors [14]. To mimic decreases in dopamine levels, we used the antagonist flupenthixol, which also works on both types of dopamine receptors non-selectively, but in the opposite way as the agonist [14]. We injected pea aphid mothers and measured the percentage of winged morphs among their offspring as a response to the treatment. To assess the generality of the results, we performed the experiments in two genetically unique pea aphid lines, ROC1 and SSC3. As it is not possible to precisely control the percentage of winged offspring produced in each experiment and because this can differ across replicates, we also performed each experiment two times (referred to as round 1 and round 2) to assess replicability. As noted above, the relevant comparisons in our study are always within experiments, between the treatment and control samples.

Injecting apomorphine significantly decreased winged morph percentages in offspring across both experimental rounds for both aphid lines (figure 1, electronic supplementary material, table S3; Wilcoxon rank-sum tests, ROC1 round 1 adjusted *p*-value = 0.0010, ROC1 round 2 adjusted *p*-value = 0.0012, SSC3 round 1 adjusted *p*-value = 0.0012, SSC3 round 2 adjusted *p*-value = 0.0090). Injecting the antagonist flupenthixol, on the other hand, produced the predicted, opposite result: it significantly increased winged percentages (figure 1, electronic supplementary material, table S3; Wilcoxon rank-sum tests, ROC1 round 1 adjusted *p*-value = 0.0090, ROC1 round 2 adjusted *p*-value = 0.0090, SSC3 round 1 adjusted *p*-value = 0.0001, SSC3 round 2 adjusted *p*-value = 0.0090).

We found no significant effects of treatments on fecundity (electronic supplementary material, figure S1, electronic supplementary material, table S4). We did, however, observe that the fecundity in the last antagonist experiment (SSC3 round 2) was noticeably lower than in other experiments (electronic supplementary material, figure S1). We do not know the reason for this. The lower fecundity of this experiment led in part to our choice of excluding replicates with less than six offspring because more restrictive filters would have led to the

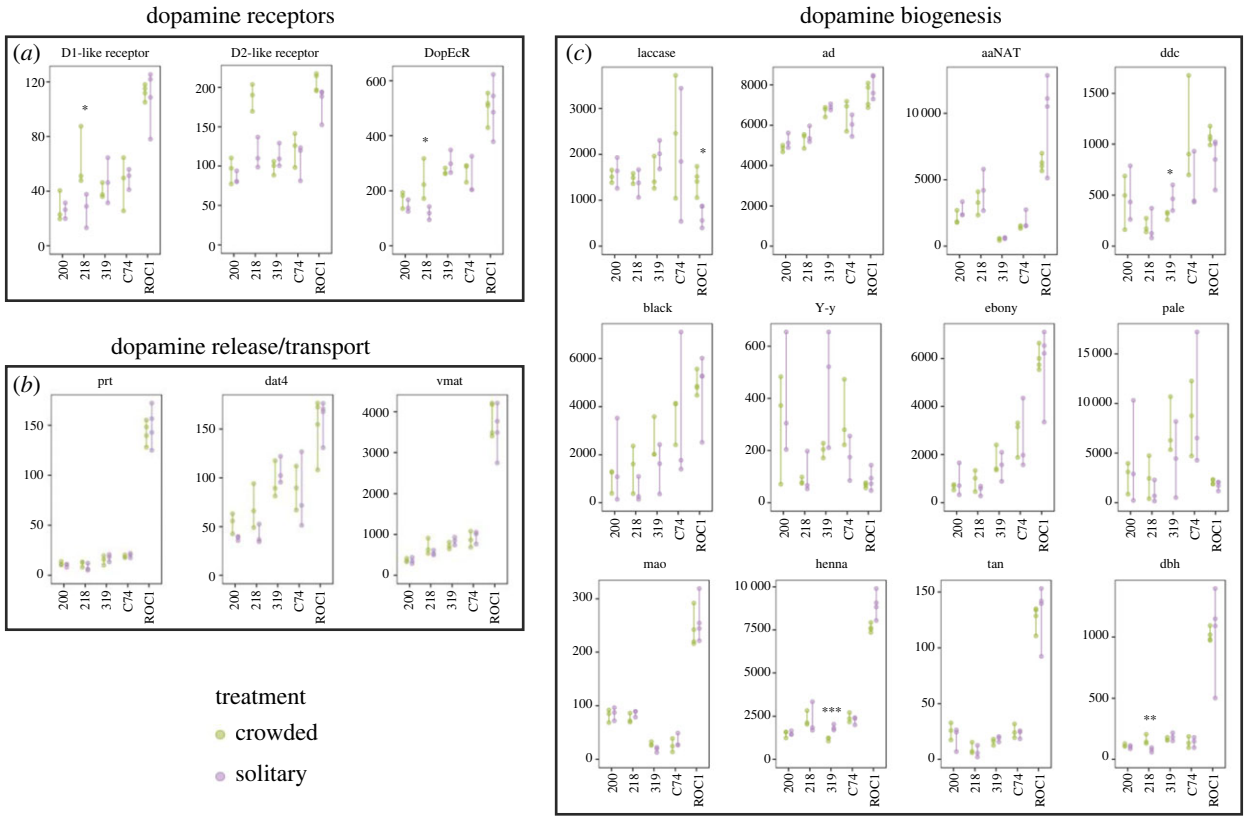

Figure 2. Dopamine-related gene expression levels generally do not differ in aphid mothers experiencing crowded versus solitary treatments. The genes are broken up into the categories of (*a*) dopamine and dopamine/ecdysteroid receptors, (*b*) dopamine release or transport, or (*c*) dopamine biogenesis. The *y*-axis shows expression level in normalized read counts, with each gene having a different axis. The *x*-axis shows the five different aphid lines. Crowded (wing-producing) or solitary (wingless-producing) treatment means aphids were kept in those conditions for 12 or 16 h (see Methods). Each point represents a biological replicate. Lines 319, C74, 200 and 218 each have three biological replicates per treatment. Line ROC1 has four biological replicates per treatment. Gene names are as in electronic supplementary material, table S2. *Adjusted *p*-value ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

elimination of the majority of replicates for this experiment. To explore how this choice affected our analyses, we re-analysed our injection data using the stricter threshold for inclusion of 10 offspring or more. We found that all winged offspring percentage comparisons remained significant except for the experiment with low fecundity, the SSC3 round 2 antagonist experiment (electronic supplementary material, table S3). Under the stricter threshold, there were only five replicates for the treatment group, strongly reducing the power of the test (electronic supplementary material, tables S1 and S3). Fecundity comparisons remained non-significant for all experiments, regardless of any filters (electronic supplementary material, table S4). We conclude that manipulating dopamine levels can robustly change the proportion of winged offspring, supporting the idea that dopamine plays an important role in the pea aphid wing plasticity.

We then used previously generated adult asexual female whole-body (minus ovaries and embryos) RNA-Seq datasets to examine the expression level of 21 genes important for dopamine metabolism and signalling in crowded (largely winged-offspring producing) versus solitary adult females (largely wingless-offspring producing). Although a few genes exhibited significant differences for individual lines (figure 2), we observed no consistent differences of any of these genes between crowded and solitary treatments across all five lines examined (figure 2; Dat1, Dat2 and Dat3 showed no expression and thus were excluded from the plots).

4. Discussion

Here we have provided evidence for the ability of dopamine to mediate the pea aphid wing dimorphism. This dimorphism is transgenerational and likely involves a multi-stage process: the pea aphid mother must sense whether she is likely to give birth in a low or high-density environment, convert that information into a molecular signal to send to the embryos, and those embryos must respond to that signal by committing to a wingless or winged developmental programme. We need to associate these events with their molecular changes to fully understand the mechanistic basis of this plasticity.

We hypothesize that dopamine signalling is important for the early part of this process, in which the pea aphid mother integrates information about the density level of her environment. Our rationale is twofold. First, pea aphid mothers in low-density environments exhibit higher dopamine titres than those in high-density environments [8], meaning that the mothers themselves likely display dopamine level differences. And second, dopamine signalling has a well-established role in linking environment-based learning and memory in multiple species (e.g. [15]). Intriguingly, in another insect, *Drosophila melanogaster*, dopamine signalling has been shown to interact with ecdysone signalling (as it does in *Drosophila* [16]), which is a known modulator of the pea aphid wing plasticity [17]. This suggests a possible cascade of information that flows from the environmental density signal, to dopamine signalling, and then to ecdysone

signalling, with the eventual output being a winged or wingless daughter.

While we have shown that dopamine signalling plays an important role in that wing plasticity, what remains especially unclear is what, if any, gene expression level changes result in changes in dopamine signalling. We detected no differences in expression levels of any dopamine-related genes. It is possible that we have not sampled the appropriate timepoints to catch relevant gene expression changes (dopamine titre levels were measured after 24 h of crowded versus solitary conditions [8], while most of the RNA-Seq data were collected at either 12 h or 16 h [8,10]). It is also possible that we are missing tissue-specific gene expression because our RNA-Seq datasets were generated from whole-body females with embryos removed, and dopamine-related expression can be tissue specific (e.g. [18]). Future experiments involving time-course analyses and focusing on dopaminergic tissues would address these issues. And finally, differences could be achieved by non-transcriptional related mechanisms, such as inactivation by oxidation or methylation (reviewed in [15]).

Our work adds to a growing literature implicating dopamine signalling in the mechanistic basis of phenotypic plasticity. For example, in grasshoppers (*Locusta migratoria*), dopamine promotes the phenotypically plastic change from solitary to gregarious behaviour [9]. In the water flea, (*Daphnia longicephala*), treatment with dopamine causes increased size and neck teeth expression [5]. In non-plastic insects, dopamine signalling is activated under stressful conditions (e.g. [19–21]). It is possible that phenotypically plastic arthropod systems have co-opted this aspect of the generalized stress response to modulate their environmentally sensitive phenotypes, much like some vertebrates have modified the corticotropin-releasing hormone stress-responsive axis to regulate phenotypic plasticity [22].

Data accessibility. All raw data and related resources are deposited and available from Dryad [23] at: https://datadryad.org/stash/share/pxNkDJvyBbO-qAntZRNT9JkhfsPGpPyCW-PnLnWrLpg.

The data are provided in the electronic supplementary material [24].

Authors' contributions. X.L.: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing—original draft, writing—review and editing; J.A.B.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This research was supported by National Science Foundation IOS award 1749514 to J.A.B.

Acknowledgements. We thank Jen Keister and Erica Culbert for their help with these experiments.

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