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Author for correspondence:

Michael P. Shahandeh

e-mail: Michael.P.Shahandeh@hofstra.edu

Laboratory and wild *Drosophila sechellia* have conserved niche specialization phenotypes

Michael P. Shahandeh^{1,2}, Liliane Abuin², Olakunle A. Jaiyesimi^{3,†}, Polpass Arul Jose^{3,4,†}, Suhrid Ghosh^{3,4,†}, Asfa Sabrin Borbora^{2,†}, Janpreet Kaur^{1,†}, Cassandra G. Extavour^{3,4} and Richard Benton²

¹Department of Biology, Hofstra University, Hempstead, NY, USA

²Center for Integrative Genomics, University of Lausanne, Lausanne, Switzerland

³Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, USA

⁴Howard Hughes Medical Institute, Chevy Chase, MD, USA

PAJ, 0000-0003-4488-7048; CGE, 0000-0003-2922-5855; RB, 0000-0003-4305-8301

Many studies investigating the proximate causes of evolutionary adaptations dissect potentially adaptive phenotypes under laboratory conditions where organisms are more easily experimentally manipulated. For such models, it remains unclear if organisms maintained long-term under laboratory conditions are representative of relatives in their natural environment. *Drosophila sechellia*, a fly endemic to the Seychelles that uses the toxic *Morinda citrifolia* noni fruit as a sole niche, has emerged as a powerful (neuro)genetic model for studying the molecular basis of ecological adaptations. Many studies have investigated the genetic and cellular basis of this species' specialization in a laboratory setting using inbred strains that were collected many decades ago and have been maintained under conditions very different from those in nature. Here, we compare the phenotypes of these strains with recently collected wild *D. sechellia* to ask if long-term laboratory maintenance has led to a loss or degradation of phenotypes associated with their specialization. We show that newly derived *D. sechellia* lines display highly similar anatomical and behavioural phenotypes to long-term laboratory strains, including noni attraction and olfactory sensory neuron numbers, toxin resistance, egg size and circadian plasticity. These results support the use of inbred, genetically manipulatable, strains to study ecological adaptation.

[†]These authors contributed equally to this study.

1. Introduction

A significant challenge to studying the genetic and cellular underpinnings of ecological adaptation is the necessity to remove individuals from their natural niche for laboratory experimentation. This means that many mechanistic studies of the development, physiology and behaviour of a wide variety of organisms are traditionally performed in conditions that differ, sometimes drastically, from an animal's natural environment. This issue is particularly pertinent for laboratory models, where organisms are brought into artificial environments, inbred and maintained at reduced population sizes for thousands of generations. This process creates several challenges in comparing laboratory strains with their wild counterparts.

One of these is the potential for adaptation to laboratory conditions, especially early during the establishment of laboratory strains. For instance, a standard *Caenorhabditis elegans* laboratory strain displays dramatic differences in social and feeding behaviours, growth and reproductive output compared with wild *C. elegans*, probably resulting from adaptation to laboratory rearing [1,2]. Moreover, several laboratory strains of *C. elegans*, and even other *Caenorhabditis* species, have convergently evolved unnatural resistance to dauer-inducing pheromones—a starvation-resistant stage of developmental arrest—which is thought to be an adaptation to laboratory culture at high densities [3]. Exacerbating this problem, it is possible for the same strains maintained in different laboratories to experience different artificial/unintended selection pressures resulting from differences between these environments (e.g. culture medium and rearing practices), creating laboratory-specific phenotypic differences for the same original laboratory strain. For instance, in the mouse model, *Mus musculus*, laboratory rearing environment influences molecular and behavioural phenotypes [4], in some cases resulting in the division of genetic isolates into substrains [5,6].

The process of creating isogenic strains through inbreeding can itself result in differences between natural and laboratory populations. For instance, inbreeding depression [7] affects phenotypic measures in the laboratory, and inbred strains, at best, provide only a small sample of the total genetic variation present in natural populations [8]. Moreover, long-term maintenance of small populations renders many deleterious mutations effectively neutral, increasing their likelihood of fixation via genetic drift, which can lead to stochastic differentiation and deterioration of isolates of the same laboratory strain over time. Indeed, laboratory-specific replicates of the commonly used *Drosophila melanogaster* isogenic strain, Canton-S, display significant defects in locomotor behaviour that seem unlikely to result from laboratory adaptation [9]. Regardless of the underlying cause(s), it is clearly justified to question if laboratory-reared strains are representative of their original populations.

This issue of phenotypic divergence upon prolonged laboratory rearing is potentially more extreme for specialist species, which display adaptation to a narrow ecological niche that is not always easily replicated in a laboratory setting. One example is *Drosophila sechellia*, which diverged from a common ancestor with *D. melanogaster* no more than 5 million years ago [10]. Despite the phylogenetic proximity and high genomic and anatomical similarity of these drosophilids [11], *D. sechellia* occupies a dramatically different ecological niche. While *D. melanogaster* is a cosmopolitan species, feeding generally on rotting fruit [12], *D. sechellia* is endemic to the Seychelles archipelago, where it specializes on the ripe noni fruit of the shrub *Morinda citrifolia* [13] (figure 1A), which is toxic to most other *Drosophila* species [15]. Moreover, in its near-equatorial habitat, *D. sechellia* is exposed to tropical conditions year-round, unlike globally distributed *D. melanogaster* [10], which experiences higher degrees of seasonal variation at high latitudes. As such, *D. sechellia* has been a focus of many comparative studies aimed at describing the genomic, developmental, physiological, behavioural and neurological evolution that underlies its niche specialization (reviewed in [16]).

Most studies on *D. sechellia* have used laboratory strains established with multiple mated females collected in 1981 [17]. With approximately 25–30 generations per year, this currently represents more than 1000 generations of isolation, inbreeding, mutation accumulation and potential adaptation to laboratory rearing practices since their establishment. Moreover, in the laboratory, these strains typically are not reared on noni fruit, but rather on standard *Drosophila* medium, only sometimes supplemented with commercially available noni juice (depending upon the practices of the laboratory and individual researcher). Even when it is used, this juice only partially resembles noni fruit chemically [18], and has limited, at most, toxicity for other drosophilids (unpublished observations). It is therefore unclear how representative these laboratory strains of *D. sechellia* are of those living in their specific niche today, or whether specialization has degraded over the course of long-term laboratory maintenance in an artificial environment. Recent field studies have sought to address similar issues in *D. melanogaster* by observing the behaviours of natural populations of flies to collect data that can be compared with

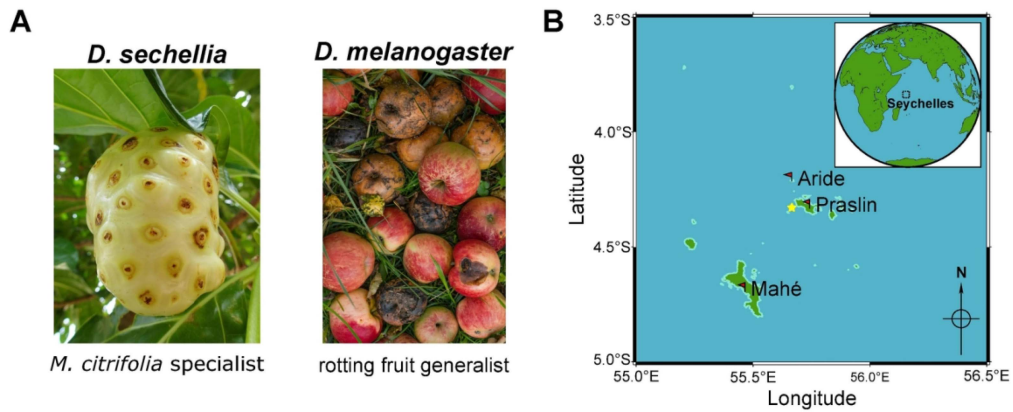


Figure 1. *Drosophila sechellia* is a *M. citrifolia* noni fruit specialist, endemic to an equatorial island archipelago. A. Host preferences of *D. melanogaster* and *D. sechellia*. B. A map of the Seychelles archipelago with specific coordinates of wild *D. sechellia* strain collection sites on three islands marked with red flags (Mahé island coordinates: 4.69424° S, 55.46580° E, Aride island coordinates: 4.21423° S, 55.66555° E and Praslin island coordinates: 4.33340° S, 55.74180° E), and Cousin island, the collection site of strains 07 and 28, marked with a yellow star. Upper-right inset shows the location of the Seychelles on the eastern hemisphere. The maps were generated using Generic Mapping Tools (GMT) version 6.6.0 [14].

laboratory generated data [19–21]. Here, we also sought answers to these questions by performing anatomical and behavioural laboratory assays on recently collected lines of *D. sechellia* from different islands of the Seychelles archipelago (figure 1B). We measured multiple aspects of *D. sechellia*'s host specialization to compare these non-laboratory and laboratory strains.

2. Results

2.1. Wild *Drosophila sechellia* collection

Drosophila sechellia flies were collected in April 2023 during a field trip to three different islands in the Seychelles archipelago (figure 1B) and used to establish isofemale lines. Strain M1311 was obtained from the grounds of the National Biodiversity Center on Mahé island. Strains A3312 and A3F311 were collected from a site on Aride island. Strains P7611 and P7621 were collected from outside Vallée de Mai on Praslin island. All strains except A3F311 were founded from single adult females collected directly from the surfaces of fallen noni fruits using a manual fly aspirator. Strain A3F311 was founded from a female that eclosed from a larval-infested fallen noni fruit collected in the field and brought back to the laboratory. Each strain was determined to be *D. sechellia* by molecular barcoding (see §5).

2.2. Olfactory preference for ripe noni volatiles

Drosophila sechellia displays a strong preference for ripe noni fruit and noni-derived acids over other substrates in open trap assays, permitting olfactory and gustatory sampling both in the laboratory [22] and field [23]. In long-range and short-range laboratory assays that exclude gustatory sampling, *D. sechellia* displays increased olfactory-mediated preference for noni and noni volatiles [18,24,25]. This strong preference for noni has been attributed, in part, to evolution of the peripheral olfactory sensory system [24,26].

To compare the phenotypes of laboratory and wild *D. sechellia*, we first examined their olfactory preference, given a choice between noni fruit juice and apple cider vinegar (a fermentation product that is highly attractive to *D. melanogaster* [27]). We tested two commonly used laboratory *D. sechellia* strains, the five new wild-caught *D. sechellia* strains and one *D. melanogaster* laboratory strain. In our trap design, flies could not physically encounter the liquid prior to making a choice, limiting the possibility for gustatory sensation (figure 2A). All seven *D. sechellia* strains, both laboratory and recently caught, displayed a significant preference for noni juice over apple cider vinegar, while *D. melanogaster* displayed a slight but non-significant preference for apple cider vinegar (figure 2B). Importantly, there were no distinguishable differences when comparing the preferences between *D. sechellia* strains, regardless of their

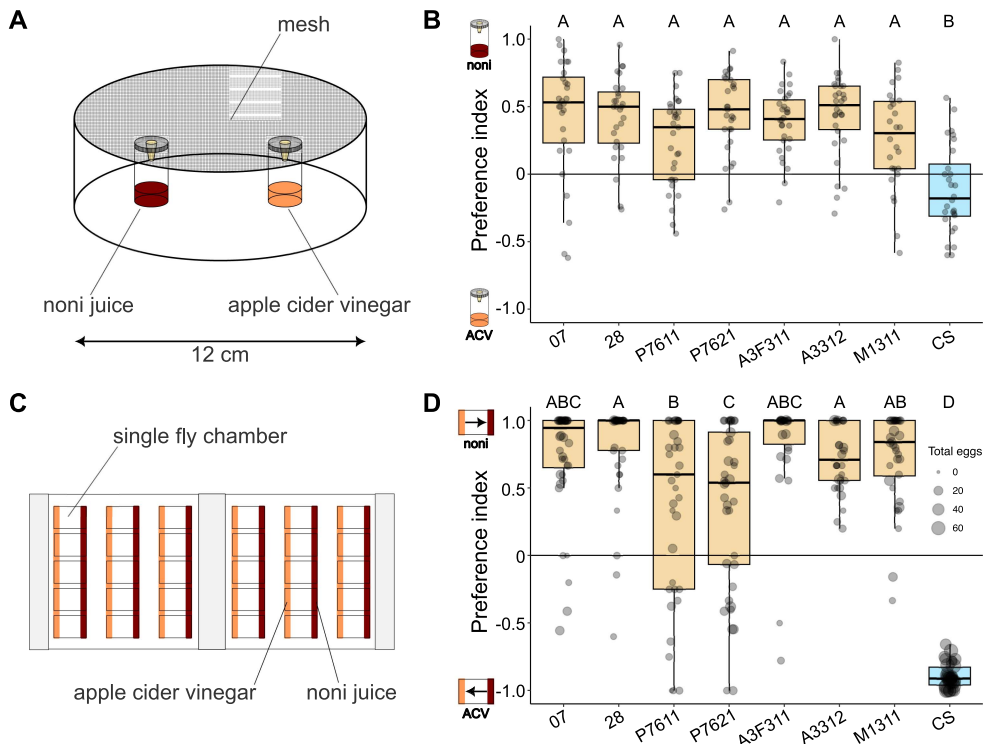


Figure 2. *Drosophila sechellia* display strong olfactory preference for noni in short-range olfactory and oviposition assays. A. Schematic of the short-range olfactory preference assay. B. Short-range olfactory preference of wild and laboratory *D. sechellia* strains (orange) and *D. melanogaster* Canton-S (CS, blue). All *D. sechellia* strains displayed a significant preference for noni juice over apple cider vinegar (all $p < 0.01$, Wilcoxon test), while CS displayed no significant preference. Sample sizes: 07 (28), 28 (28), P7611 (31), P7621 (29), A3F311 (30), A3312 (30), M1311 (26) and CS (28). C. Schematic of the single-fly oviposition preference assay. D. Single-fly oviposition preference for the same strains. Point size is scaled by the total number of eggs laid per female. All *D. sechellia* strains displayed a significant preference for noni juice over apple cider vinegar (all $p < 0.01$, Wilcoxon test), while CS displayed a significant preference for apple cider vinegar ($p < 0.001$, Wilcoxon test). Sample sizes: 07 (40), 28 (39), P7611 (37), P7621 (37), A3F311 (30), A3312 (30), M1311 (36) and CS (34). For panels B and D, letters above boxplots indicate pairwise significant differences (all $p < 0.01$, Wilcoxon test followed by Bonferroni correction).

collection date. However, all *D. sechellia* strains displayed significantly different preference indices from *D. melanogaster*.

2.3. Oviposition preference for noni substrates

Upon successful location of a host fruit, flies will feed, engage in social behaviours with other flies they encounter and reproduce [19]. *Drosophila sechellia* consistently selects ripe noni or noni media for oviposition over other substrates [22,23,28,29]. This inherent preference to oviposit on ripe noni has been attributed to olfactory receptor tuning changes [28] and gustatory preference differences [30].

To compare the oviposition preferences of our recently caught and laboratory *D. sechellia* strains, we replicated a single-fly oviposition choice assay [28,31]. Briefly, we offered individual gravid females a choice between two oviposition media of equivalent texture, made with noni juice or apple cider vinegar, over a 24 hour period. During this period, flies were able to sample each media using tactile, gustatory and olfactory cues (figure 2C). In this assay, all *D. sechellia* strains displayed a significant preference for ovipositing on noni media over apple cider vinegar media, while our *D. melanogaster* laboratory strain displayed a significant preference for ovipositing on apple cider vinegar media (figure 2D). All *D. sechellia* strains were statistically distinguishable from *D. melanogaster*. In this assay, we observed significant variation among our *D. sechellia* strains with respect to the strength of their preference for ovipositing in noni media, but this did not reflect a consistent distinction between laboratory and wild *D. sechellia* (figure 2D).

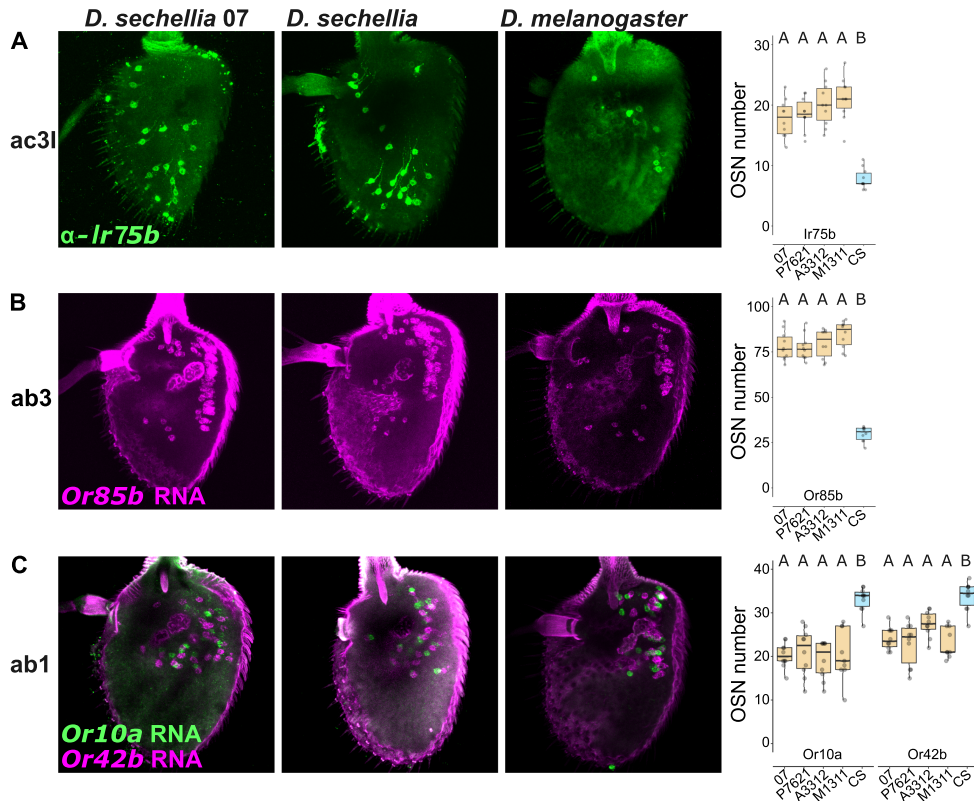


Figure 3. Olfactory sensory neuron population expansion and contraction in *D. sechellia*. A. Left: example images of Ir75b OSNs in ac3I sensilla of the third antennal segment of laboratory *D. sechellia* (07), wild *D. sechellia* (P7612) and *D. melanogaster* (CS). Right: quantifications of Ir75b OSNs in laboratory *D. sechellia* (07), *D. melanogaster* (CS) and three wild *D. sechellia* strains, one from each island (P7612, A3312 and M1311). B. Left: example images of Or85b OSNs in ab3 sensilla for the same strains as in panel A. Right: quantifications of Or85b OSNs for the same strains as in panel A. C. Left: example images of Or10a and Or42b OSNs labelled in ab1 sensilla for the same strains as in panel A. Right: quantifications of Or10a and Or42b OSNs for the same strains as panels A. For A–C, letters above boxplots indicate pairwise significant differences (all $p < 0.05$, Wilcoxon test followed by Bonferroni correction). Ten antennae were imaged and quantified for each sensillum type per strain.

2.4. Olfactory sensory neuron population expansion and contraction

The species-specific host location and oviposition behaviours of *D. sechellia* are thought to be due in part to increases in the numbers of olfactory sensory neurons (OSNs) detecting characteristic noni volatiles [32], namely hexanoic acid-sensing Ir75b OSNs in antennal coeloconic 3I (ac3I) sensilla and Or22a and Or85b OSNs in antennal basiconic 3 (ab3) sensilla, which detect methyl esters and 2-heptanone, respectively [18,24,33].

To determine whether the expansions described in laboratory strains are a conserved phenotype of *D. sechellia* in the wild, we used immunofluorescence or RNA fluorescence *in situ* hybridization to label specific OSN populations in one laboratory strain each of *D. melanogaster* and *D. sechellia*, as well as three of the wild-caught *D. sechellia* strains (P7612, A3312 and M1311). Across both laboratory and wild-caught *D. sechellia* strains, we observed a consistent twofold increase in Ir75b OSNs relative to *D. melanogaster* (figure 3A). Similarly, by labelling Or85b OSNs, we detected a consistent expansion of ab3 sensilla (figure 3B), with *D. sechellia* strains exhibiting a threefold increase in OSN number compared with *D. melanogaster*.

In addition to these OSN population expansions, several sensilla and/or OSN types are reduced in *D. sechellia*. For example, ab1 sensilla, which house Or10a and Or42b expressing OSNs, display a nearly twofold reduction in number relative to *D. melanogaster* [24,33]. In *D. melanogaster*, Or42b neurons are required for attraction to apple cider vinegar [27], so their decrease in *D. sechellia* plausibly reflects a lower significance of this sensory stimulus. Labelling of Or10a and Or42b neurons revealed a consistent reduction in all tested *D. sechellia* strains compared with *D. melanogaster* (figure 3C).

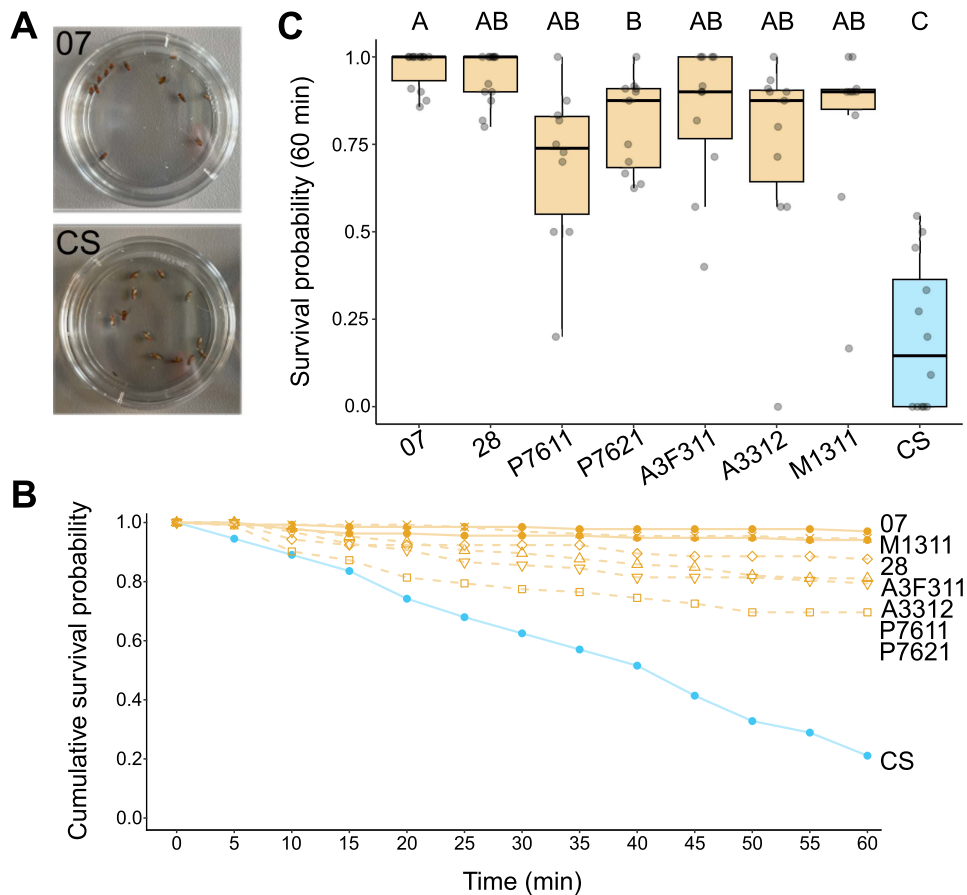


Figure 4. *Drosophila sechellia* adult females display high resistance to the noni toxin octanoic acid. A. Photographs of a single replicate of laboratory *D. sechellia* (07) and *D. melanogaster* (CS) strains following 60 minutes of exposure to pure octanoic acid. B. Cumulative survival probabilities (mean proportion of living flies across all replicates) of laboratory *D. sechellia*, wild *D. sechellia* and *D. melanogaster* CS is shown for 5-minute increments over a 60-minute exposure duration. C. Final survival probability (proportion of living flies after 60 minutes of exposure to octanoic acid) for each replicate is shown for wild and laboratory *D. sechellia* strains and *D. melanogaster*. Letters above boxplots indicate pairwise significant differences (all $p < 0.05$, Wilcoxon test followed by Bonferroni correction). Sample sizes: 07 (14), 28 (13), P7611 (10), P7621 (11), A3F311 (11), A3312 (11), M1311 (10) and CS (12).

2.5. Adult noni toxin resistance

As part of its specialization on noni, *D. sechellia* has evolved a high resistance to octanoic acid, an abundant noni chemical [34] that accounts for this fruit's toxicity for other drosophilids [35,36]. The mechanistic basis of adult octanoic acid resistance in *D. sechellia* is still poorly understood, but genome-wide association and gene expression studies identify a complex genetic architecture [37] highlighting candidates involved in cuticle formation [38–40], enzymatic detoxification [41–43] and metabolism [44,45].

To compare the resistance to octanoic acid of our laboratory-adapted strains with that of recent collections, we used a plate-based assay [37]. Adult males and females were combined with 2 μ l of pure octanoic acid in a Petri dish to ensure exposure to its volatiles and scored every 5 minutes for loss of consciousness over 60 minutes (figure 4A). When we calculated the cumulative survival probability across 10 replicates for our *D. sechellia* and *D. melanogaster* strains, we observed a consistent species difference: for all *D. sechellia* strains, more than 60% of females (figure 4B) and males (supplementary material, figure S1A) remained conscious, while approximately 20% of *D. melanogaster* females and no males did. Comparing the proportion of surviving flies at 60 minutes between strains for both females (figure 4C) and males (supplementary material, figure S1B), all *D. sechellia* strains were significantly different from *D. melanogaster*. For females, we detected some variation among our wild-caught *D. sechellia* (figure 4C), but this reflected slightly lower octanoic acid resistance levels compared with the laboratory strains.

2.6. Fecundity and egg size

In addition to adult resistance to noni toxins, *D. sechellia*'s eggs must be able to develop on this toxic fruit. The eggs of *D. sechellia* are larger (approx. 50% volume) than those of *D. melanogaster* [46] and it has been hypothesized that this phenotype represents part of the mechanism permitting their successful development on noni. This potential increase in resource allocation toward individual eggs is further hypothesized by some to have resulted in a trade-off with overall fecundity [33,34], due at least in part to a decrease in total ovariole number, though it remains to be determined if and how this trait evolved during *D. sechellia*'s specialization [47]. While the molecular basis of reduced ovariole number is unknown, association mapping studies have indicated a complex genetic architecture [48,49], and developmental studies have identified differences in somatic gonadal precursor cell number [50] and insulin signalling [51] that contribute to this trait.

To examine these phenotypes in wild-caught flies, we quantified reproductive output for mated females, ovariole number and egg size. We used the single-fly oviposition assay described above to determine the total reproductive output of a single female over a 24-hour period. Comparing the total eggs produced per female, irrespective of oviposition media, we found that all *D. sechellia* strains were consistently distinguishable from *D. melanogaster*, laying approximately fivefold fewer eggs (all $p < 0.001$, figure 5A). Similarly, all *D. sechellia* strains, irrespective of collection date, had fewer ovarioles (approx. twofold reduction) than our *D. melanogaster* strain (figure 5B). Finally, all *D. sechellia* strains laid larger eggs than *D. melanogaster* (figure 5C), with the average volume of *D. sechellia*'s eggs being 45% greater than those of *D. melanogaster* Canton-S. For these latter two phenotypes, we did not observe any significant differences between any *D. sechellia* strain (figure 5B,C).

Taken together, the decreased fecundity, decreased ovariole number and increased egg size we document is similar to previously described reproductive divergence between laboratory strains of *D. melanogaster* and *D. sechellia* [46,48,52], and consistent with the hypothesis of an evolutionary trade-off between reproductive output and resource allocation [30].

2.7. Reduced circadian plasticity

Finally, in addition to *D. sechellia*'s many adaptations to its host fruit, it was recently discovered to be specialized to aspects of its equatorial environment, notably photoperiod (day length) [53]. *Drosophilid* flies typically display a bimodal pattern of circadian activity (with some exceptions), where individual flies time their peak morning and evening activity with sunrise and sunset [54]. Flies living at high latitudes display an ability to plastically adjust the timing of these peaks to match seasonal variation in day length, with the degree of plasticity correlating with population latitude [53,55,56]. *Drosophila sechellia* are found exclusively close to the equator, where day length varies minimally throughout the year [10]. As such, these flies are far less able to delay the timing of their evening peak activity to match longer days that would be experienced at higher latitudes [53]. This reduced plasticity has a polygenic basis, including divergent regulation of the circadian neuropeptide, *Pigment dispersing factor* [53].

We used the *Drosophila* activity monitor system to compare the activity patterns of our strains under both equatorial and high-latitude summer conditions [57]. We first recorded male flies of each strain for 7 days under 12 : 12 hour light-dark (LD) (equatorial) conditions at 25°C (figure 6A). All strains of both species displayed a characteristic peak of activity surrounding subjective dawn and dusk, separated by an afternoon period of reduced activity. We next took the average activity for each fly per strain during the last 4 days of this recording and annotated the timing of the evening activity peak (figure 6B). *Drosophila melanogaster* and all the laboratory and wild-caught *D. sechellia* strains displayed a high synchronicity of their evening peak time with the timing of subjective dusk (12 hours).

We next modified the photoperiod for these flies, recording them for an additional 7 days under 16 : 8 hour LD (high-latitude summer) conditions (figure 6C). As before, we used the average activity from the last 4 days of this recording period from each fly to determine the timing of the evening activity peak under extended photoperiod conditions (figure 6D). As previously described [53], we observed a clear species difference in evening peak plasticity, where our *D. melanogaster* laboratory strain was able to delay its evening peak time significantly longer than any of our *D. sechellia* strains. On average, *D. melanogaster* Canton-S was able to delay its evening peak to approximately 15 hours, just 1 hour shy of subjective dusk. By contrast, our *D. sechellia* strains' average evening peak time ranged from approximately 13 to 13.67 hours. Comparing laboratory and wild-caught *D. sechellia* strains identifies a single wild-caught strain from Praslin island (P7611) with a slightly elevated evening peak time relative to just one of our laboratory strains, *Dsec07*. There is, therefore, no consistent discernable difference in circadian

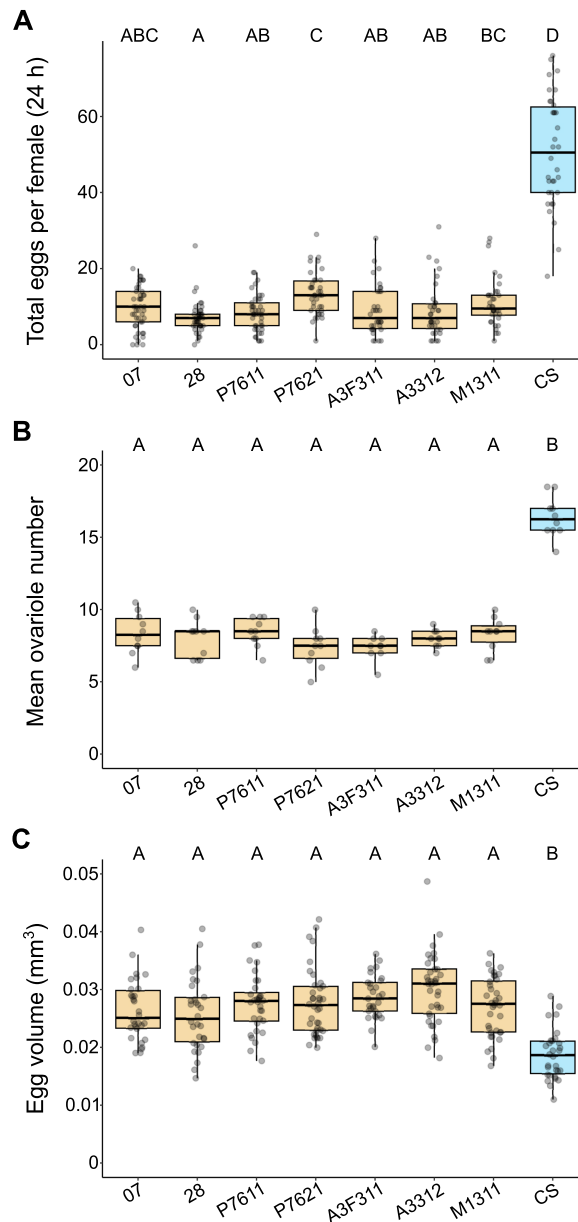


Figure 5. *Drosophila sechellia* lay fewer, larger eggs and display a reduced ovariole number compared with *D. melanogaster*. A. The total eggs laid per female in a 24-hour period is shown for laboratory *D. sechellia*, wild *D. sechellia* and *D. melanogaster* CS. Total eggs laid per female was obtained from the experiments shown in figure 2C,D. B. The volume of individual eggs for the same strains as panel A. Sample sizes: 07 (30), 28 (30), P7611 (32), P7621 (38), A3F311 (29), A3312 (37), M1311 (36) and CS (30). C. The mean number of ovarioles per ovary for individual adult female flies is shown for the same strains as panels A and B. Sample sizes: 07 (10), 28 (10), P7611 (10), P7621 (10), A3F311 (9), A3312 (9), M1311 (10) and CS (10). For A–C, letters above boxplots indicate pairwise significant differences (all $p < 0.05$, Wilcoxon test followed by Bonferroni correction).

plasticity between laboratory and wild-caught *D. sechellia* strains. This is unsurprising because, for this phenotype, standard laboratory fly culture conditions (12 : 12 hour LD cycle at 25°C) more accurately reflect equatorial conditions than the variable conditions at high latitudes in the wild.

3. Discussion

3.1. Long-term laboratory strains retain specialism-associated phenotypes

Here, we investigated whether long-term laboratory maintenance of *D. sechellia* strains have resulted in anatomical and/or behavioural divergence from their wild counterparts. Our results unequivocally

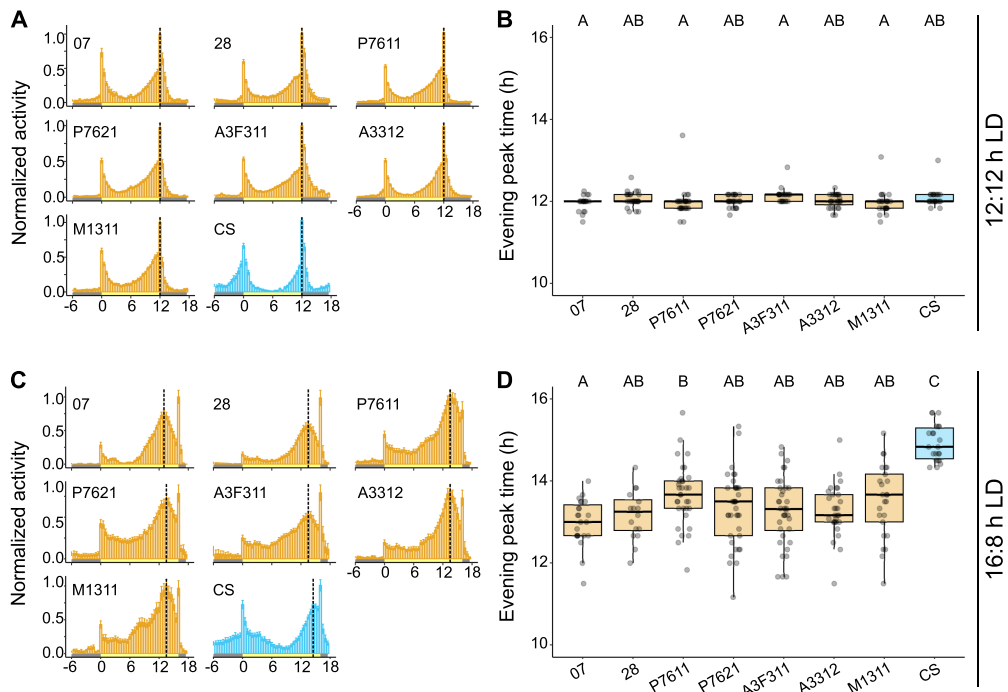


Figure 6. *Drosophila sechellia* displays reduced circadian plasticity during long days. A. Maximum normalized average activity plots for laboratory *D. sechellia*, wild *D. sechellia* and *D. melanogaster* CS under a 12 : 12 hour photoperiod. B. Mean evening peak time for the last 4 days of a 7 day period under 12 : 12 hour photoperiod conditions is shown for individual flies for the same strains as panel A. Sample sizes for panels A and B: 07 (21), 28 (28), P7611 (29), P7621 (28), A3F311 (24), A3312 (27), M1311 (26) and CS (24). C. Maximum normalized average activity plots for the same strains as panels A and B under a 16 : 8 hour photoperiod. D. Mean evening peak time for the last 4 days of a 7 day period under 16 : 8 hour photoperiod conditions is shown for individual flies for the same strains as panels A–C. Sample sizes for panels C and D: 07 (23), 28 (16), P7611 (34), P7621 (37), A3F311 (36), A3312 (29), M1311 (25) and CS (22). For A and C, histograms display maximum normalized activity of flies in 30 minute bins across a 24 hour period. Error bars denote standard error of the mean. Grey and yellow bars along the x-axis represent lights-off and lights-on in the incubator, respectively. Vertical dashed lines represent the average evening peak time. For B and D, letters above boxplots indicate pairwise significant differences (all $p < 0.05$, Wilcoxon test followed by Bonferroni correction).

indicate that *D. sechellia* laboratory strains do not display signs of a loss or degradation of the behavioural or anatomical phenotypes previously associated with this species' specialism. In general, the lack of laboratory-specific evolution we document might result from the nature of these phenotypes themselves, whose genetic underpinnings are likely to be fixed in this small specialist population with low genetic diversity [58]. The creation of inbred lines is probably more representative of the genetic make-up of the larger population compared with broadly distributed generalist species that have higher standing genetic variation and which might exhibit local adaptation [59,60]. These findings justify the use of inbred laboratory strains to study the mechanisms through which species specialize to a specific ecological niche. We discuss below our results on a phenotype-by-phenotype basis.

Despite not being reared in the presence of noni fruit for hundreds of generations, *D. sechellia* laboratory strains show a strong retention of preference for noni-based substrates in both olfactory and oviposition assays, along with the increases/decreases in OSN number associated with these preferences. For this study, we maintained our *D. sechellia* strains on standard *Drosophila* media supplemented with a paste made with commercially available noni juice. It is possible that this approximation of noni fruit is sufficient to maintain noni preferences during laboratory culture. However, chemical comparison of this juice with ripe noni fruit reveals differences in the relative proportions of specific chemicals, such as octanoic and hexanoic acids [18]. Likewise, our laboratory *D. sechellia* strains were originally sourced from the *Drosophila* Species Stock Center (DSSC), where they were previously maintained for years on a cornmeal media (DSSC Director Patrick M. O'Grady, personal communication), so it seems unlikely that our more recent maintenance on noni juice media would be sufficient to maintain preference for noni. Given the apparent lack of selection to maintain noni preferences, and the many known examples of laboratory adaptation, why, then, have the *D. sechellia* laboratory strains not evolved novel preferences for laboratory media? It is likely that in this specialist species, the genetic underpinnings of host

preference are fixed, and that these inbred strains have simply not been reared in the laboratory for long enough to evolve altered preferences *de novo*. Additionally, a laboratory environment probably does not select strongly for altered preferences: in standard culture vials, flies have no need to forage and are not given a choice between media, and therefore, have little option to exert (or need for) a preference. Thus, any chemosensory preferences might be effectively neutral in a laboratory setting, and such phenotypes would only degrade slowly over time via the accumulation of random mutations and genetic drift.

This hypothesis might also explain the persistence of octanoic acid resistance in laboratory populations. There is no obvious benefit to retaining octanoic acid resistance in the laboratory, as these flies never encounter it. However, as long as the cost of retaining octanoic acid resistance is not very large, selection also would not favour its loss in a small laboratory population, again rendering it a neutral phenotype. Alternatively, there might be hidden selective advantages. For example, cuticle changes that confer octanoic acid resistance might also result in decreased desiccation risk, which could still be highly favoured in a laboratory setting where humidity is not always precisely controlled. Thus, instances of strong laboratory selection on one effect of the phenotype might maintain another.

We also show that the reduced fecundity and ovariole number previously described in *D. sechellia* laboratory strains is indeed representative of the phenotypes of wild-caught flies. Laboratory environments typically select for high fecundity through high-density rearing practices [3]. Our laboratory strains do not appear to have either responded to or experienced this selection pressure. However, if the hypothesis of a trade-off in *D. sechellia* of reduced fecundity with increased egg size is correct, for laboratory strains to evolve increased fecundity, they might also have to evolve reduced egg size, which presents a more complicated evolutionary trajectory [36,37]. In this respect, reduced fecundity of *D. sechellia* might represent an evolutionary dead-end. Future studies exploring differences in maternal investment to directly test this hypothesis seem warranted.

Laboratory *D. sechellia* strains have also retained their circadian specialization to an equatorial environment. Considering most laboratories maintain flies under equatorial conditions with respect to photoperiod and temperature, this is unsurprising. In this context, it is notable that *D. melanogaster* maintained at equatorial conditions have not lost their capacity for photoperiodism, generally supporting the idea that laboratory strains maintain, at least over these time scales, aspects of local adaptation to their collection site.

3.2. Phenotypic variation among recently collected *Drosophila sechellia*

While we did not observe a loss or degradation of specialism phenotypes in long-term laboratory *D. sechellia* strains, we did observe variation in some of these phenotypes among our recently collected strains. One pattern that stands out is a reduction in oviposition preference for noni media paired with reduced octanoic acid resistance in the strains collected from Praslin island relative to the others. Notably, one of these strains, P7621, also displays the greatest fecundity. It is possible that these differences reflect population structure, where flies found on one island might be both genetically and phenotypically differentiated from flies on the others. Indeed, a recent population genetic study found that flies sampled from Praslin island formed a separate cluster in both principal components and structure analysis when compared with flies sampled from Mahé, La Digue and Marianne (the latter two were not sampled in this study) [61]. One potential driver of population structure could be variation in hybridization and introgression rates between *D. sechellia* and its close relative in sympatry, *D. simulans* [61,62]. Future endeavours may expand sampling paired with genome sequencing to better elucidate the structure of *D. sechellia* populations on the Seychelles for improved comparison with the phenotypic variation we describe here.

4. Conclusion

Finally, while it is most parsimonious to assume that the mechanisms underlying specialism-associated traits are conserved between laboratory and wild-caught strains—as supported here by the observed expansions and contractions of OSN populations—future studies should further investigate in our wild-caught strains the previously described evolution of gene expression [41,42,44,53,63,64], the nervous system [18,24,25,28,30,53,64,65] and the reproductive system [50,51]. Regardless, our data demonstrate robustness in *D. sechellia*'s specialism-associated phenotypes, further supporting the power of this species as a model system to uncover ecologically relevant genetic adaptations. Such conclusions are also

encouraging for the use of other laboratory models of specialization, such as *Drosophila erecta* [66,67] and *Drosophila mojavensis* [68].

5. Methods

5.1. *Drosophila sechellia* wild-caught strain maintenance

Following field collections in April 2023, isofemale *D. sechellia* lines were established and maintained in the Extavour laboratory on *Drosophila* media (0.8% agar, 2.75% yeast, 5.2% cornmeal and 11% dextrose) without noni juice at 25°C, 65% relative humidity and a 12 : 12 hour LD cycle, from May to November 2023. From November 2023, flies from each strain were reared in the Benton laboratory on *Drosophila* media (0.6% agar, 5.8% yeast, 5.8% wheat and 10% fruit juice) supplemented with noni juice paste (Raab Vitalfood Bio noni juice combined with instant *Drosophila* medium, Carolina #173 210). From January 2025, flies were reared in the Shahandeh laboratory on *Drosophila* media (0.9% agar, 2.6% yeast, 6.25% cornmeal and 6.25% molasses) supplemented with noni juice (Healing Noni juice combined with instant *Drosophila* medium, Carolina #173 210). In both the Benton and Shahandeh laboratories, flies were maintained in non-overlapping 2-week generations at 25°C, 50% relative humidity and a 12 : 12 hour LD cycle. To limit the potential for laboratory adaptation and inbreeding to occur, all behavioural analyses reported herein were conducted within a year of collection (by May 2024) in the Benton laboratory. Quantifications of ovariole number, egg size (Shahandeh laboratory) and OSN number (Benton laboratory) were conducted from July to December 2025.

5.2. *Drosophila* laboratory strain maintenance

The laboratory strain of *D. melanogaster* (Canton-S, RRID:BDSC_64349) was maintained on *Drosophila* media (recipes above) in the Benton and Shahandeh laboratories in non-overlapping 2-week generations at 25°C, 50% relative humidity and a 12 : 12 hour LD cycle. The laboratory strains of *D. sechellia* (*Dsec.07*, DSSC 140210248.07; *Dsec.28*, DSSC 140210248.28) were maintained under the same conditions on *Drosophila* media supplemented with noni juice paste (as described above for each laboratory).

5.3. Species identification by molecular barcoding

Wild-caught flies were identified as *D. sechellia* using molecular barcoding of portions of the *mitochondrial cytochrome c oxidase (mt:Col)* gene, using COI-218F (5' CAACATTTATTTTGATTTTGG 3') and COI-3037R (5' TYCATTGCACTAATCTGCCATATTAG 3') primers, by standard methods. In brief, genomic DNA was extracted from individual flies, amplified via polymerase chain reaction (PCR) using OneTaq 2X Master Mix (includes 0.62 units Taq; New England BioLabs #M0480) and purified using Monarch PCR & DNA Cleanup Kit (New England BioLabs #T1030L). The resulting products were subject to Sanger sequencing (Bioscience, Inc.), using COI-218F primer. Raw sequence data were analysed using Chromas Lite v. 2.6.6 (Technelysium Pty Ltd., Australia) and sequences were compared with those in GenBank to identify the species. Flies were identified as *D. sechellia* if the *mt:Col* amplicon showed at least 99.5% sequence identity with GenBank accession MK659840.1, which is the *mt:Col* sequence of *D. sechellia* voucher DSECH20161109 complete mitochondrial genome. *mt:Col* amplicon sequences from the strains described in this study were deposited in GenBank (accession numbers: M1311, PX502215; A3312, PX502212; A3F311, PX502211; P7611, PX502214; and P7621, PX502213).

5.4. Olfactory trap assay

The olfactory trap assay was replicated from previous studies investigating *D. sechellia*'s short-range olfactory preference [18,25]. In brief, 25 3–5-day-old mated females were anaesthetized with ice and added to a 12 cm circular plastic Tupperware containing two olfactory traps prior to sealing the tops with a fine mesh secured by rubber band (figure 2A). Each trap contained 300 µl of either noni juice (Raab Vitalfood) or apple cider vinegar (Denner brand) mixed with Triton-X (final concentration 0.2%). Flies can enter the traps through a pipette tip puncturing the cap but are unable to find the opening to escape after selecting an odour source. Traps were stored for 24 hours at 25°C and approximately 60% humidity under red light, after which the number of flies in each trap or remaining untrapped animals (live and dead) were

counted. We excluded from our statistical analyses any traps where more than 25% of flies remained untrapped (supplementary material, figure S2). We calculated preference indices as: (number of flies in the noni juice trap – number of flies in the apple cider vinegar trap)/number of living flies, both trapped and untrapped. We used a Wilcoxon rank-sum test to test individual preference indices for differences from 0 (no preference) and pairwise Wilcoxon tests to compare preference indices between strains. For both, *p*-values were corrected for multiple comparisons using the Bonferroni method.

5.5. Two-choice single-fly oviposition assay

To measure individual oviposition preferences, we used a single-fly chamber assay where 30 3–5-day-old mated females are housed individually in Plexiglas cells, where they can sample two oviposition media [28,31] (figure 2C). We provided a choice between two substrates: a 0.67% agar solution made with noni juice, or an agar solution of the same density prepared with apple cider vinegar. Prior to assay, females were housed in high densities with males for 3 days to ensure they were gravid. The chambers were housed at 25°C and approximately 60% humidity for 24 hours under a 12 : 12 hour LD cycle. At the end of the assay, flies were removed and the number of eggs present on each substrate was recorded for each chamber. We calculated preference indices as: (number of eggs laid on the noni media – the number of eggs laid on apple cider vinegar)/total number of eggs laid. We used a Wilcoxon rank-sum test to test individual preference indices for differences from 0 (no preference) and pairwise Wilcoxon tests to compare preference indices between strains. For both, *p*-values were corrected for multiple comparisons using the Bonferroni method.

5.6. Histology and imaging

Hybridization chain reaction RNA fluorescence *in situ* hybridization (HCR-FISH) was performed, as described in [69]. RNA probes for *Or10a* (accession number NM_078567.5), *Or42b* (NM_078900.2) and *Or85b* (NM_079555.2), amplifiers and buffers were purchased from Molecular Instruments (HCR™ RNA FISH v. 3.0). Probe sequences correspond to *D. melanogaster* *Or* genes, but have greater than or equal to 95% identity with orthologous *Ors* in *D. sechellia*.

Immunofluorescence for Ir75b was performed as described previously [18], using anti-Ir75b [25] diluted 1 : 500. Secondary anti-guinea pig antibody conjugated to Alexa Fluor 488 (A11073, Invitrogen AG) was diluted 1 : 1000.

Images were acquired using a Zeiss confocal microscope LSM 710 with a 40× objective and processed in *ImageJ*. Neurons were counted manually using the ImageJ MultiPoint tool.

5.7. Octanoic acid resistance assay

To measure adult resistance to octanoic acid, we used a previously described plate assay [37,44] (figure 3A). In brief, 10 male or female flies were briefly anaesthetized with CO₂ and placed in a 60 mm diameter × 15 mm high Petri dish to which 2 µl of pure octanoic acid had been previously added to the centre of the lid. Upon recovery from anaesthesia, the flies were scored every 5 minutes for loss of consciousness for 60 minutes. To test for differences between strains, we compared the proportion of surviving flies at the final time point using pairwise Wilcoxon tests, followed by post hoc correction for multiple comparisons.

5.8. Ovary dissection and counting

Three–five-day-old, mated females (reared at 25°C, approx. 60% relative humidity, 12 : 12 hour LD) were anaesthetized on CO₂ and sorted by sex. Whole flies were fixed for 2 hours at room temperature in 4% paraformaldehyde in PBS containing 0.3% Triton X-100 (PBS-TX; 1 ml per approx. 30 flies) on a nutator. Samples were washed three times for 5 minutes in PBS-TX and ovaries were dissected in PBS-TX under a stereomicroscope. To enhance contrast for ovariole visualization, each set of dissected ovaries was briefly dipped in crystal violet solution (K1184, APEXBio). Each ovary was gently teased apart in PBS-TX on a ceramic slide using an eyelash probe to separate ovarioles without rupturing the ovary. Ovarioles were identified as individual strings of progressively developed egg chambers. Both ovaries from each female were counted and averaged to obtain mean ovarioles per ovary.

5.9. Egg collection and size measurements

Oviposition plates were prepared as 5% sucrose and 2% agarose in distilled water, and diluted with noni juice or apple cider vinegar to a final concentration of 1%. Plates were poured into 60 mm diameter Petri dishes and cooled to room temperature. Groups of mated females (3–5 days old; conditioned with males at high density for 3 days prior) were transferred to fresh plates and allowed to oviposit for approximately 20 hours at 25°C, approximately 60% relative humidity and 12 : 12 hour LD cycle. For imaging, a dissecting scope was used to locate eggs; individual eggs were placed into a single file line on the plate on their dorsal side. We photographed each plate along with a ruler for scale using a Nikon Z50 DX 16–50 mm on a tripod approximately 24 cm above the plate's surface. Egg size was quantified in Fiji/ImageJ (<https://imagej.net/ij/>). For each egg, length (L) and width (W) were measured using the straight-line tool. We calculated total egg volume using a previously established formula [70]: $V = 1/6\pi W^2 L$ for approximately 30 eggs of each strain. Two observers independently estimated egg volume using the same images to control for observer bias (figures 5C; supplementary material, figure S3), as a completely blind study was not possible owing to differences in the colour of substrate used for each species. Note, a second observer found greater variation in egg size among *D. sechellia* strains, but these differences were not consistent across observers (supplementary material, figure S3). Both observers, however, found consistent differences between all *D. sechellia* and *D. melanogaster* strains.

5.10. *Drosophila* activity monitoring

We used the *Drosophila* Activity Monitor system to record the daily activity patterns of our strains under both equatorial (12 : 12 hour LD) and high-latitude summer (16 : 8 hour LD) photoperiod conditions [71], replicating experiments used to describe reduced circadian plasticity in *D. sechellia* [53]. Briefly, we exposed individual 3–5-day-old males first to 7 days of an equatorial photoperiod, followed by 7 days of a high-latitude summer photoperiod. We used a previously developed algorithm to quantify the timing of each fly's evening peak using the last 4 days of each 7-day period, discarding the first 3 days as an acclimation period [53]. We compared evening peak time between our strains using pairwise Wilcoxon tests, followed by correction for multiple comparisons.

To estimate the circadian period of each strain, we acclimated flies to a 12 : 12 hour LD cycle for four days before subjecting them to 5 days of constant darkness conditions. We used the constant darkness behavioural data to calculate the duration of each strain's free-running period with the R package Rethomics, which was also used for all circadian data visualization [72]. We compared periods across strains with pairwise Wilcoxon tests subjected to post hoc correction for multiple comparisons.

5.11. Statistical analysis and data visualization

All statistical analyses were conducted using the 'stats' package (v. 4.6.0) in R (v. 4.4.2) [73]. Data were visualized using 'ggplot2' [74]. For boxplots in all figures, the bold line represents the median value, with the length of the box displaying the interquartile range. Whiskers signify the last quartiles excluding outliers.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All data presented in this manuscript and the code used for statistical analysis and data visualization are available at [75]. mt:CoI amplicon sequences from the strains described in this study were deposited in GenBank (accession numbers: M1311, PX502215; A3312, PX502212; A3F311, PX502211; P7611, PX502214; P7621 and PX502213).

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Author's contributions. M.P.S.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, writing—review and editing; L.A.: data curation, investigation, methodology, project administration, writing—original draft, writing—review and editing; O.A.J.: data curation, investigation, methodology, writing—original draft, writing—review and editing; P.A.J.: data curation, investigation, methodology, visualization, writing—original draft, writing—review and editing; S.G.: data curation, investigation, methodology, visualization, writing—review and editing; A.S.B.: investigation, methodology, writing—original draft, writing—review and editing; J.K.: investigation, methodology, writing—original draft, writing—review and editing; C.G.E.: funding acquisition, project administration, resources, supervision, writing—review and editing; R.B.: conceptualization, funding acquisition, project administration, resources, supervision, 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.

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