Short Article

Developmental Cell

A Temperature-Dependent Switch in Feeding Preference Improves *Drosophila* Development and Survival in the Cold

Highlights

- Fruit flies switch their feeding preference from yeast to plants at low temperature
- Flies need PUFAs from plants to increase membrane lipid unsaturation in the cold
- Dietary plant lipids maintain membrane fluidity and improve survival in the cold

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In Brief

How do cold blooded animals cope with temperature changes? Fruit flies manage this by changing their diet. At low temperature, they switch from yeast to plants as a preferred food source. Plants provide polyunsaturated fatty acids they need to tune membrane lipid composition and increase membrane fluidity at low temperature.

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A Temperature-Dependent Switch in Feeding Preference Improves *Drosophila* Development and Survival in the Cold

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SUMMARY

How cold-blooded animals acclimate to temperature and what determines the limits of their viable temperature range are not understood. Here, we show that *Drosophila* alter their dietary preference from yeast to plants when temperatures drop below 15°C and that the different lipids present in plants improve survival at low temperatures. We show that *Drosophila* require dietary unsaturated fatty acids present in plants to adjust membrane fluidity and maintain motor coordination. Feeding on plants extends lifespan and survival for many months at temperatures consistent with overwintering in temperate climates. Thus, physiological alterations caused by a temperature-dependent dietary shift could help *Drosophila* survive seasonal temperature changes.

INTRODUCTION

Cold-blooded animals develop and reproduce successfully over a wide temperature range. *Drosophila melanogaster* provides a powerful genetic system in which to probe the mechanisms underlying temperature acclimation. The genetic regulatory networks underlying its growth and metabolism have been well studied, and there has been extensive work on its ecology and population genetics. Populations of *Drosophila melanogaster* are thought to survive year-round in temperate and tropical climates and are found in habitats with a wide range of average seasonal temperature fluctuations (Hoffmann, 2010).

The response of *Drosophila* adults to temperature extremes has been studied in the lab using *Drosophila* stocks recently established from wild populations and then maintained on lab diets. While successful development is restricted to the range between 12°C and 30°C, *Drosophila* can withstand a few hours at higher (38°C) and lower (−2°C) temperature extremes (Hoffmann, 2010; Hoffmann et al., 2002). These limits can vary somewhat depending on the geographical area from which the flies are isolated and can be extended by "hardening," i.e., pre-exposure to high or low temperatures (Kelty and Lee, 1999, 2001; Sejerkilde et al., 2003). Since temperatures even in temperate climates can exceed these limits, it seems likely that *Drosophila* must behave in a way that enables them to avoid extreme temperatures in their natural environments, that key ingredients in temperature acclimation are missing from the lab, or both. It is unclear exactly where or how *Drosophila melanogaster* overwinter in the wild.

Seasonal changes in temperature pose interesting challenges for cold-blooded animals because temperature strongly influences the rates of biochemical reactions and the physical properties of matter, such as the phase transition-dependent temporal and spatial organization of the cytoplasm (Banani et al., 2017) and cellular membranes (Sezgin et al., 2017; Veatch et al., 2008). Temperature alters the fluidity, ion permeability, and phase behavior of lipid bilayers (Hazel, 1995), and maintaining membrane biophysical properties within a functional range is essential for life at changing temperature. Phase separation underlies formation of membrane micro-domains involved in protein sorting and signaling (Cebecauer and Holowka, 2017; Lingwood and Simons, 2010). Furthermore, membrane fluidity can modulate the activity of membrane proteins—for example, the Na^+/K^+ ATPase, which maintains ion homeostasis and membrane potential (Bhatia et al., 2016; Cornelius et al., 2015; Wu et al., 2001). Excessive rigidity of membranes at low temperatures may account for the loss of K^+ homeostasis and membrane potential associated with chill coma in insects (Macmillan and Sinclair, 2011). Bacteria, fungi, plants, and some animals have been observed to alter their membrane composition when the temperature changes to at least partially compensate for temperature—a phenomenon known as homeoviscous or homeophasic adaptation (Hazel, 1995). Decreasing temperature is associated with increasing fatty acid unsaturation, shorter fatty acid chain length, and an elevation in the PE/PC ratio—all of which are predicted to increase membrane fluidity (Cossins et al., 1977; Cossins and Prosser, 1978; Kemp and Smith, 1970).

The physiological changes that occur in *Drosophila melanogaster* during rapid cold hardening and other types of cold

exposure have been explored using metabolomics, lipidomics, proteomics, and transcriptomics (Colinet et al., 2013; Koštál et al., 2011; MacMillan et al., 2009; Overgaard et al., 2007; Overgaard et al., 2005, 2006; Qin et al., 2005; Zhang et al., 2011). Several studies report increases in levels of sugars in response to cold (Colinet et al., 2016; Koštál et al., 2011; Overgaard et al., 2007), although this is not always seen (Kelty and Lee, 2001; MacMillan et al., 2009). Proline accumulates in the cold and has been suggested to act as a cryoprotectant (Koštál et al., 2011). However, the fluidity of *Drosophila* membranes from animals acclimated to different temperatures has never been measured, and the extent to which homeoviscous adaption occurs is unclear. Other cold-blooded animals such as fish increase average fatty acid unsaturation by about 20% upon cold acclimation (Hazel, 1984). However, the evidence for this in *Drosophila* is equivocal. Many studies report no significant changes in average fatty acid unsaturation in the cold (Colinet et al., 2016; Koštál et al., 2011; MacMillan et al., 2009; Ohtsu et al., 1998; Overgaard et al., 2008). Others observe increases in average unsaturation on the order of 1% (Overgaard et al., 2005, 2006). Only one study of *Drosophila* cold acclimation reports larger changes in unsaturation (Cooper et al., 2012). The sources of this variability are not understood.

One key feature that varies in these studies is diet, which strongly influences the degree of fatty acid unsaturation in membrane phospholipids in *Drosophila* (Carvalho et al., 2012). Like that of yeast, their key food source, the *Drosophila* genome encodes only a single Δ9 desaturase, suggesting they cannot introduce double bonds into fatty acids beyond the Δ9 position. Plants can desaturate fatty acids not only at Δ9 but also the Δ12 and Δ15 positions. Flies, like humans, must obtain these fatty acids from the diet in order to produce lipids with polyunsaturated fatty acids (PUFA) (Carvalho et al., 2012; Randall et al., 2015).

Wild *Drosophila melanogaster* are thought to feed primarily on the yeasts present on decomposing fruit. Olfactory and gustatory cues attract them to yeasts, both as a food source and as a substrate for egg laying. Although they can detect plant compounds derived from leaves or unripe fruit, these are less attractive than yeast or are even aversive (Becher et al., 2012; Hoang et al., 2015; Koštál et al., 2012; Stensmyr et al., 2003; Versace et al., 2016). It is unclear to what extent they consume plants and, if so, whether it is important.

We recently developed two different food recipes containing exclusively yeast or plant material. These foods contain similar proportions of calories derived from protein, lipid, and carbohydrate but differ in their lipid composition (Brankatschk et al., 2014; Carvalho et al., 2012). Yeast glycerolipids contain saturated or mono-unsaturated fatty acids (MUFAs) with 14–18 carbon units, while plant lipids also contain longer and PUFAs. Plant food contains phytosterols, while yeast food contains fungal sterols (Buttke et al., 1980; Carvalho et al., 2012). Larvae fed with these two different diets have different lipidomes, and only plant-fed larvae accumulate significant amounts of phospholipids containing PUFAs (Carvalho et al., 2012).

The different lipids present in plant and yeast food influence not only membrane lipid composition but also developmental rate, fertility, and lifespan (Brankatschk et al., 2014; Carvalho et al., 2012). Plant-fed animals develop more slowly than yeast-fed animals, and adults live longer on a plant diet. These differences in developmental rate and lifespan are due to changes in systemic insulin/IGF (insulin-like growth factor)-like signaling (IIS). Yeast lipids, but not plant lipids, cause lipoproteins to accumulate on specific CNS neurons that connect to insulin producing cells (IPCs) (Brankatschk et al., 2014). When this happens, these neurons activate IPCs, causing them to release *Drosophila* insulin-like peptides (Dilps). Dilps elevate systemic IIS, speed development, increase fertility, and shorten lifespan (Brankatschk et al., 2014; Garofalo, 2002; Giannakou and Partridge, 2007).

Why should yeast-derived lipids elevate IIS independent of the caloric content of the diet? We speculated that such a mechanism might have evolved to allow *Drosophila* to maximally exploit summer blooms of yeasts in the wild. Interestingly, distinct alleles of the *Drosophila* insulin receptor and other components of the insulin signaling pathway have been shown to vary in frequency according to latitude and even according to season (Fabian et al., 2012; Paaby et al., 2014). We wondered whether plant-derived food components might have important functions under different conditions. For example, since *Drosophila melanogaster* cannot produce lipids with PUFAs unless they consume plants, we wondered whether plant food might help flies adjust their membrane biophysical properties to survive low temperatures in the winter.

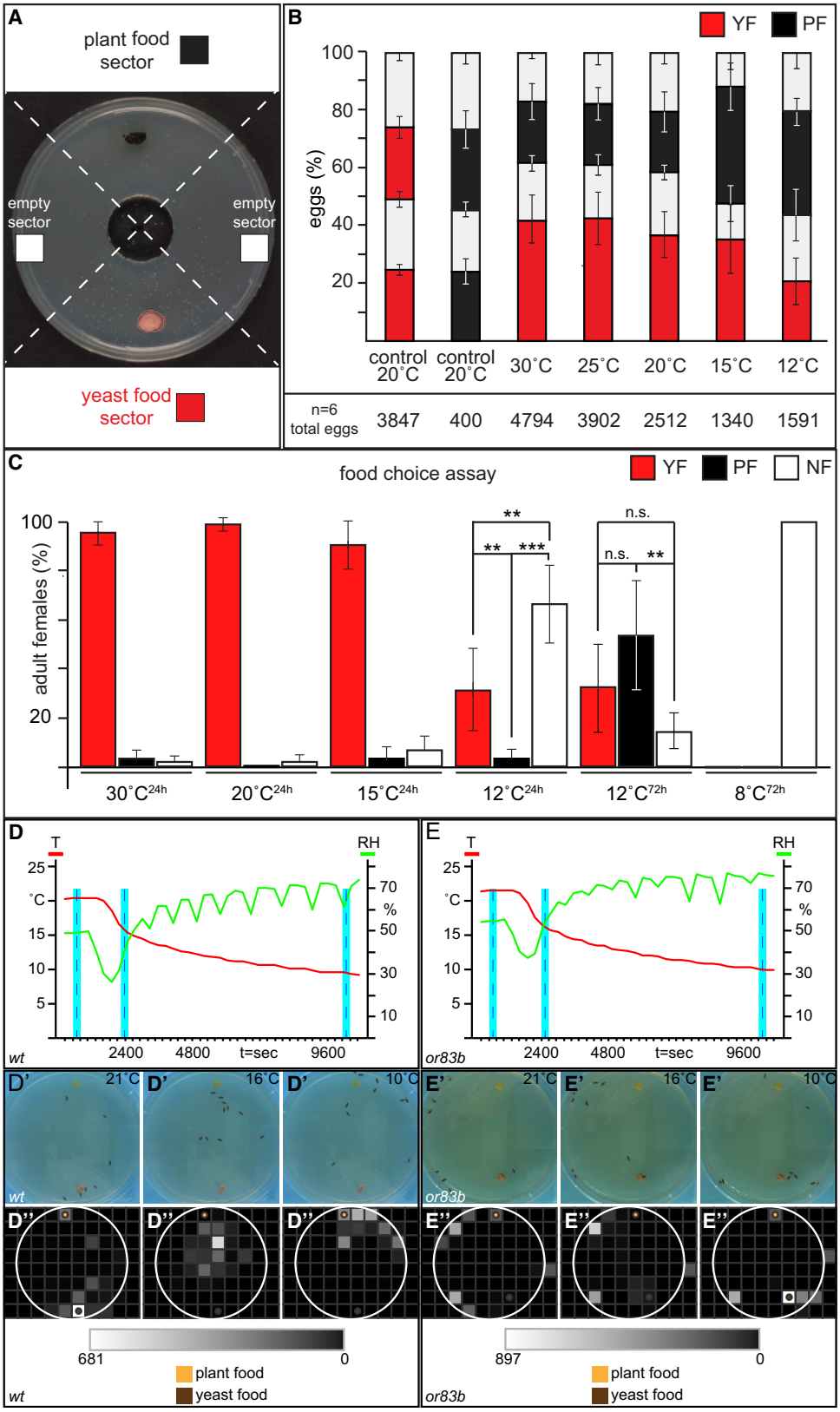
RESULTS

Drosophila Choose Plant Food over Yeast Food at Low Temperatures

Yeast-derived olfactory cues attract *Drosophila* and stimulate feeding and egg laying (Becher et al., 2012; Hoang et al., 2015; Koštál et al., 2012; Stensmyr et al., 2003; Versace et al., 2016). Although *Drosophila* clearly prefer yeast to plant-derived compounds at moderate temperatures, we wondered whether this preference might be temperature dependent. Indeed, when females of the wild-type strain OregonR are shifted to 15°C, they begin to lay eggs near plant food rather than yeast food (Figures 1A, 1B, S1A, and S1B), although they still feed on yeast (Figures 1C and S1C–S1E). At 12°C, they shift their feeding preference to plant food after a short feeding hiatus (Figures 1C and S1C–S1E).

The feeding and egg laying assays require several days to perform. To investigate how rapidly temperature influences attraction to different foods, we presented flies with a choice of plant or yeast food and filmed them as the temperature decreased from 21°C to 11°C over the course of 3 hr (Figures 1D–1D″; Video S1). As expected, flies spent more time near yeast food at 21°C. As the temperature reached 16°C (after about 40 min), flies tended to occupy regions in the middle of the plate—away from both the plant and yeast food. By the time the temperature reached 11°C, flies tended to cluster near plant food. Thus, the relative attractiveness of yeast and plant food changes rapidly as temperature drops.

We wondered whether olfactory cues were important for low temperature food choice. To address this, we performed the same experiment using *orb83* mutant flies. Orb83 is needed for dendritic localization of odorant receptors and its loss disrupts many odorant responses (Larsson et al., 2004). Flies mutant for *orb83* spend more time near the rim of the plates and do not



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congregate around either plant or yeast food at any temperature (Figures 1E–1E″; Video S2). This suggests that the preference for plant food depends either on plant-specific odorants that become attractive at low temperature or on a changed and aversive response to compounds derived from yeast.

Plant- and Yeast-Fed Larvae Develop over Different Temperature Ranges

To investigate the effects of diet on temperature-dependent survival, we quantified the fraction of larvae that pupariated on yeast and plant food at different temperatures. While 90% of yeast-fed larvae pupariate between 20°C and 30°C, only half pupariate at 15°C and none at 12°C. In contrast, plant-fed larvae efficiently pupariate between 15°C and 25°C, and a substantial number pupariate even at 12°C (Figure 2A). Thus, feeding with plants improves low temperature development. Larvae (unlike adults) do not change their feeding preference at low temperature—given a choice between plant and yeast food at 12°C–15°C, most larvae eat yeast and die (Figure 2B). Thus, if *Drosophila* females exhibit a similar temperature-dependent preference for plants as egg laying substrates in the wild, it would improve survival of their progeny below 15°C.

Consuming Plant Food Increases Cold Resistance of Adult Flies

We next examined whether feeding with yeast or plant food influenced adult survival at different temperatures. Independent of diet, lifespan lengthens as temperature drops from 30°C to 12°C but then shortens as temperature drops further to 8°C (Figures S2A–S2E), consistent with previous reports (Alpatov and Pearl, 1929; Loeb and Northrop, 1917). At all temperatures except 30°C, plant-fed flies live longer than yeast-fed flies. This suggests that the lifespan-extending effects of reduced IIS in plant-fed flies disappear at high temperature. Remarkably, plant-fed flies live up to 6 months when kept at 12°C—long enough for adults to survive through the winter.

Previous studies have shown that membrane potential and K^+ homeostasis are compromised as temperatures drop toward those that induce chill coma, causing flies to become uncoordinated (Hazell and Bale, 2011; MacMillan et al., 2015; Overgaard and MacMillan, 2017). To investigate whether a plant diet could improve coordination at low temperatures, we raised flies on plant or yeast food and transferred them to either 15°C, 12°C, or 8°C for 72 hr. We then measured the time required to climb out of a plastic tube at the respective temperatures (Figures 2C and S1H; Videos S3, S4, S5, S6, S7, and S8). Both yeast and plant-fed flies are mobile at 15°C. At 12°C, the mobility of yeast-fed flies is reduced, but that of plant-fed flies is unaffected. At 8°C, yeast-fed flies are completely immobile, while plant-fed flies are only partially compromised. Thus, plant food helps maintain normal motor function at 12°C and below.

Below about 3°C, flies enter cold coma. Below freezing, survival is typically a matter of hours (Hoffmann et al., 2002). To investigate the effect of diet at these temperatures, we fed OregonR flies plant or yeast food at 21°C–23°C and then asked how long they were able to survive at temperatures between 3° and −2°C (Figures S2F–S2H). Plant feeding almost doubles the median survival time at these temperatures. Overall, a plant diet ameliorates the effects of low temperature on coordination and survival.

Plant Food Improves Survival Outside in Fall and Winter

We wondered how diet might affect development and survival under more natural conditions where the temperature fluctuates and day-length changes. We therefore quantified larval development and adult survival of plant- and yeast-fed animals placed on the MPI-CBG rooftop in Dresden, Germany, between September 18th, 2015 and January 12th, 2016. During this time, diurnal temperature fluctuations ranged from 23°C/12°C to −1°C/−9°C (Figure 2D).

On September 18th, we allowed 20 female and 15 male OregonR flies per vial to lay eggs outside for 3 days and then monitored larval and pupal development over time. Pupariation of yeast-fed larvae ceased shortly after the night-time temperature dropped to −3°C on October 12th, and none of these animals emerged as adults (Figure 2E). In contrast, although plant-fed females laid 6-fold fewer eggs than yeast-fed females over the same time period (Figure S1F), more pupae formed in plant food vials, and pupae began to eclose as adults at the beginning of November (Figure 2E). These adults displayed the darker pigmentation characteristic of winter morphs (Clusella Trullas et al., 2007; David et al., 1990) (Figure S1G). Thus, plant food,

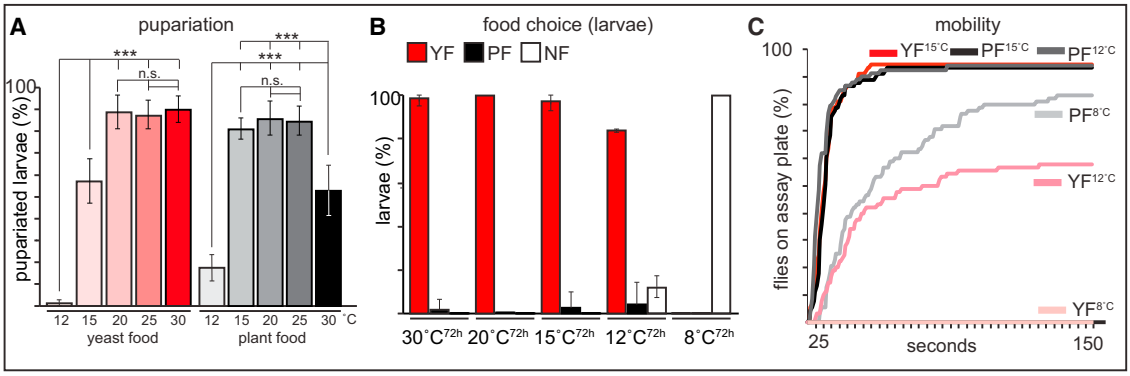
Figure 1. Food Preference Is Temperature Dependent

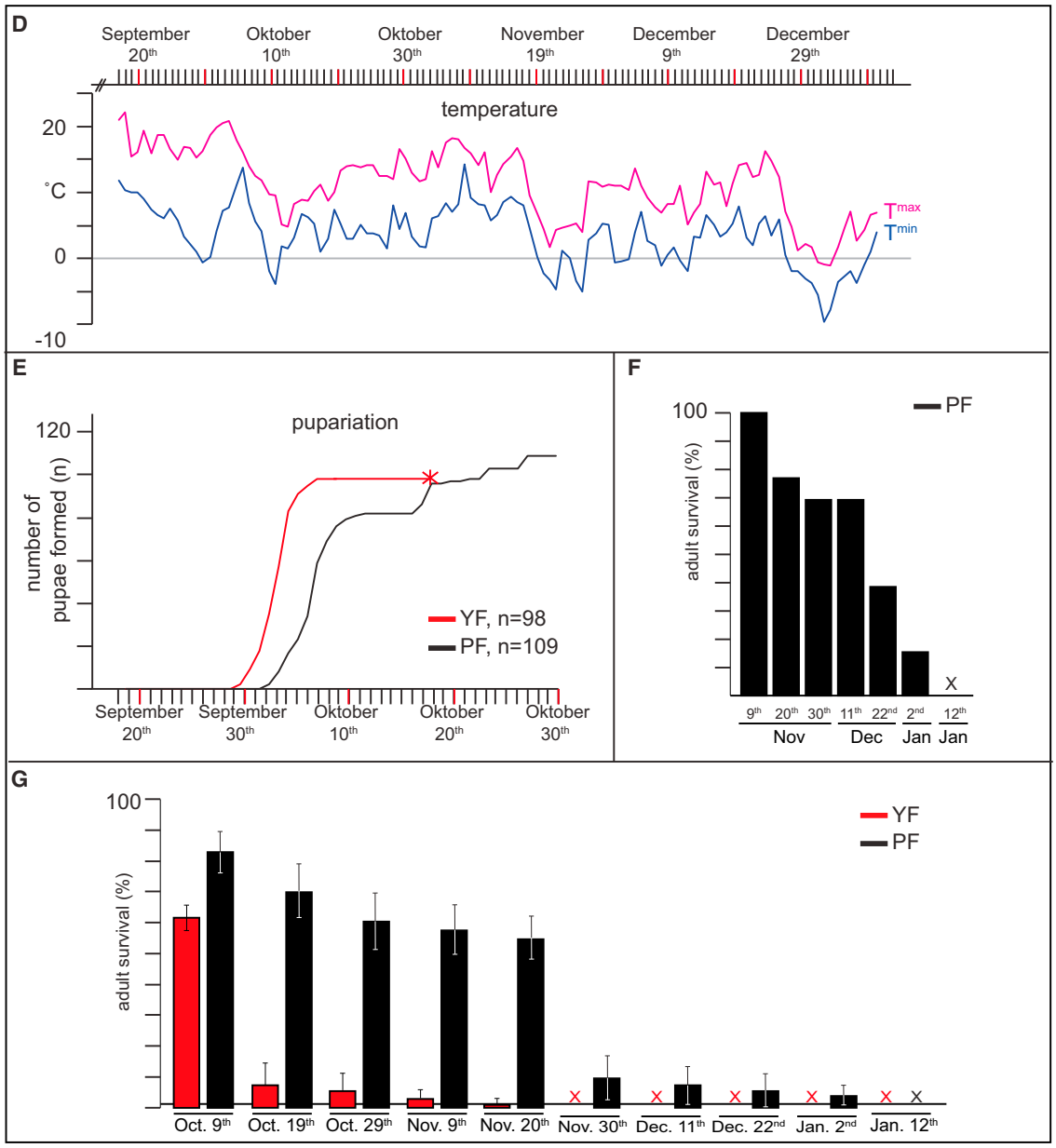
(A) Females were allowed to lay eggs on plates divided into four sectors. Two sectors contained patches of either yeast or plant food and two intervening sectors were left empty.

(B) Percentage of eggs laid in each of the four plate sectors at the indicated temperatures, along with the total number of eggs counted from six independent experiments are shown. Within each bar, red indicates plate sectors containing yeast, black indicates plate sectors containing plant food, and white indicates plate sectors with no food. As controls, we quantified eggs laid on plates containing identical foods on both sides. Error bars show standard deviation. The panel shows adjusted p values obtained from the ANOVA followed by Tukey procedure performed separately on the proportions of eggs laid either near yeast food or near plant food at different temperatures.

(C) Percentages of females that ingested yeast food (red), plant food (black), or no food (white) at the indicated temperatures after the indicated times (in hours). Error bars show standard deviation. Adjusted p values were obtained from ANOVA followed by Tukey procedure. For the experiments at 12°, the figure displays significant differences with asterisks: ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant. For the experiments at 30°C, 20°C, and 15°C, the values comparing yeast food with either no food or plant food were all $p < 0.001$. There were no significant differences between no food and plant food at these temperatures.

(D–E″) Panels depict amount of time that wild-type OregonR (D–D″) and *or83b* mutant (E–E″) flies spend near yeast and plant food as the temperature is reduced from 20°C to 10°C. Ten flies (seven females, three males) were placed on plates containing yeast food (brown) and plant food (yellow) on opposite sides in a temperature-control chamber and continuously filmed (one frame/s) as the temperature dropped. (D) and (E) show measured temperature (red) and relative humidity (green) over time. The number of flies occupying each grid position on the food plates was quantified and accumulated throughout the 4-min time windows indicated by the aqua lines in (D) and (E). Occupancy is color-coded in D″ and E″.





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but not yeast food, supports *Drosophila* development even when daily temperature fluctuations drop below freezing.

We next examined outdoor survival of adults raised on yeast or plant food and then placed outside on September 18th (Figure 2G). Most yeast-fed flies died between October 9th and October 19th, when the temperature fell to −3°C. In contrast, 70% of plant-fed flies survived. About 10% of plant-fed flies survived a cold spell on November 19th–29th, when temperatures fluctuated between 5°C and −5°C for 5 days (Figures 2D and 2G). The rest died between January 2nd and 12th, when day- and nighttime temperatures dropped to −1°C/−9°C for 3 days (Figures 2D and 2G). We also monitored survival of flies that emerged in November after developing outdoors on plant food. These survived the cold snap between November 19th and 29th better (70% versus 10%) than flies that had been raised inside at 21°C–23°C (Figures 2F and 2B). Thus, feeding adult OregonR flies on plant food, but not yeast food, allows them to survive outdoor conditions in Dresden until midwinter, and cold resistance is further enhanced when animals develop at such temperatures (see also Figure S2E). OregonR was established 90 years ago from flies caught in Roseburg, Oregon, where the average minimum nighttime temperature in January is 2°C. Our data suggest that feeding on plant food could support overwintering of these flies in such a climate.

Plant Lipids Increase Membrane Fluidity at Low Temperatures

What features of plant food promote low temperature survival? We considered whether plant-derived PUFAs, which neither yeast nor *Drosophila* can produce, might be necessary for homeoviscous adaptation (Hazel, 1995). To ask whether the lipid fraction of plant food was responsible for larval cold resistance, we devised diets containing the lipid-depleted water-soluble fraction of yeast food (Carvalho et al., 2012; Carvalho et al., 2010), supplemented by lipid extracts of plant or yeast food. We then quantified pupariation of larvae raised on these diets at different temperatures (Figure 3A). Neither food was as effective as whole yeast or plant food at supporting pupariation at any temperature—even at 20°C, only 40%–60% of larvae pupariated (compared to 80%–90% on full media). Nevertheless, plant and yeast lipids had strikingly different capacities to support larval development at different temperatures. At 15°C, larvae pupariated seven times as often when fed plant lipids. Thus, plant lipids are a key factor in the low-temperature resistance of plant-fed animals.

We next asked whether membrane biophysical properties change when *Drosophila* larvae are raised at different temperatures and whether such changes depend on diet. To monitor membrane properties in the absence of membrane proteins, we prepared liposomes from lipid extracts of feeding third instar larvae (from which guts had been removed to eliminate food lipids) and monitored C-Laurdan emission. C-Laurdan is a hydrophobic fluorescent dye that shifts its emission depending on the water content of lipid bilayers, a proxy for lipid packing and membrane order (Dinic et al., 2011; Harris et al., 2002). In this way, we measured how membrane order varied with temperature in liposomes derived from plant- and yeast-fed larvae raised at 12°C, 20°C, and 30°C (Figures 3B–3D, S2I, and S2J).

These studies showed that plant-fed larvae decrease membrane order when raised at 12°C, compared to 20°C or 30°C, while yeast-fed larvae do not (Figures S2I and S2J). Interestingly, 12°C is the temperature at which yeast-fed larvae fail to develop (Figure 2A) and at which yeast-fed adults become uncoordinated (Figure 2C). These data suggest that plant-derived dietary lipids are important for homeoviscous adaption at low temperatures.

Lipidomic Responses to Diet and Temperature

In order to assess what lipidomic changes account for altered physicochemical membrane properties of plant- and yeast-fed larvae at low temperatures, we prepared lipid extracts from feeding third instar larvae living at 10°C, 13°C, 20°C, and 30°C (see STAR Methods) and performed shotgun lipidomics. Principal-component analysis (PCA) (Kassambara and Mundt, 2015) revealed that the first two principal components (PCs) accounted for 52% (PC1) and 16% (PC2) of the lipidomic variance between these samples (Figure 3F). PC1 mainly accounts for variance due to diet, while PC2 accounts for variance caused by temperature. Examining the topology of samples along the

Figure 2. Plant Food Improves Larval Development and Adult Coordination at Low Temperatures

(A) Percentages of larvae that successfully pupariate at specified temperatures when fed with yeast food (red scale) or plant food (gray scale) are shown. Error bars show standard deviation. Adjusted p values of the differences among temperatures within each diet are represented as follows: *** = $p < 0.001$, n.s. = not significant.

(B) Larval food preference was assayed using colored food (as shown for adults in Figures S1C and S1D). Plot shows the percentages of larvae choosing yeast food (red), plant food (black), or no food (white) at the indicated temperatures and time intervals. Error bars show standard deviation.

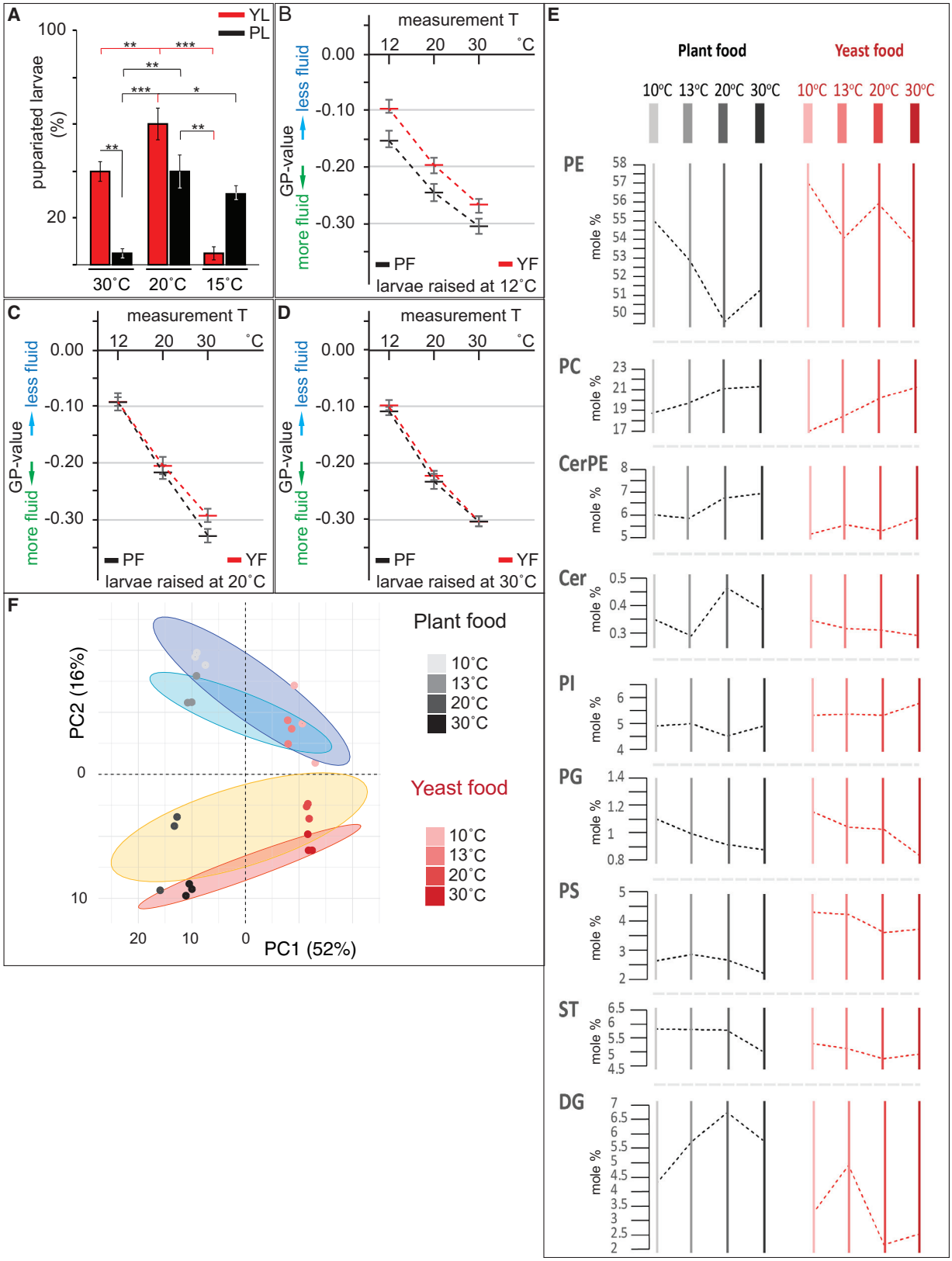
(C) Flies were raised at 21°C–23°C on either yeast or plant food and then kept for 72 hr at 15°C, 12°C, or 8°C. These flies were placed on motility assay plates (shown in A) in a water bath at the corresponding temperatures and filmed over the next 3 min. The length of time required for flies to crawl out of the tube and reach the plate was quantified in triplicate for each food and temperature condition. Red scale indicates yeast food; gray scale indicates plant food.

(D) Maximum and minimum temperatures (T^{max} magenta, T^{min} blue) for Dresden, Germany (51.03 N, 13.78 E, 119 m above sea level) from September 18th, 2015 through January 12th, 2016.

(E) Eggs laid by yeast- or plant-fed flies (kept outdoors) were collected September 18th–21st and then transferred to fresh food vials. Visual inspection of these vials confirmed our previous observation that yeast-fed females normally lay 10-fold more eggs than plant-fed females (see Figures S1A–S1C). However, practical considerations prevented us from counting the number of eggs laid by flies on the roof. Larval development and the number of pupae formed in each vial were tracked outdoors every 2 days until November 1st (lower panel). The plot shows the number of pupae formed by yeast-fed (red) or plant-fed (black) larvae over time.

(F) Panel shows the survival of flies from the population that developed on plant food outside and eclosed between October 20th and November 6th.

(G) Flies were raised on yeast or plant food at 21°C–23°C. Twenty females and fifteen males (aged between 14 and 21 days) transferred into 9 fresh food tubes and placed outdoors (roof of the MPI-CBG) on September 18th. After collecting eggs for 3 days (see B), flies were inspected for viability and transferred to new food vials every 10 days. Shown is the percentage of flies surviving on the rooftop when fed with plant food (black) and yeast food (red), as of different dates. Error bars indicate standard deviation.



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PC2 axis reveals that the lipidomes of plant-fed animals vary more in response to temperature than those of yeast-fed animals.

We first examine lipidomic differences contributing to diet-dependent variation (PC1). At all temperatures, the lipidomes of plant-fed larvae contain phytosterols rather than fungal sterols, and their phospholipids and triacylglycerides (TAGs) are longer and more unsaturated than in yeast-fed larvae (Figures S3C–S3E), consistent with our previous findings at 25°C (Carvalho et al., 2012). In plant-fed larvae, phospholipids with 3 or more double bonds (which must contain one fatty acid with at least 2 double bonds) comprise about 33% of the membrane lipidome (without TAGs). In yeast-fed larvae, they represent less than 6% (Figure S3D). TAGs are also more unsaturated when larvae feed on plant food (Figure S3E). These differences support the idea that *Drosophila* depend on dietary PUFAs to produce such lipids.

We next examine the lipidomic differences that contribute to temperature-dependent variation (PC2). Some lipidomic responses to temperature occur on both diets, and some are diet dependent. Both plant- and yeast-fed larvae increase the PE:PC ratio as temperature decreases (Figures 3E and S3, CB), consistent with previous findings (Colinet et al., 2016; Cooper et al., 2012; Cooper et al., 2014; Koštál et al., 2011; Overgaard et al., 2008). At low temperatures, both plant- and yeast-fed animals produce shorter CerPE species and replace species containing saturated fatty acids with fatty acids containing 1 double bond (Figure 4). Both changes to CerPE would be predicted to increase membrane fluidity in the cold.

In contrast, temperature-dependent glycerophospholipid fatty acid unsaturation depends strongly on diet. As temperature decreases, only plant-fed larvae decrease PE and PI species with 1 double bond and increase those with 2 (Figure 4). Furthermore, while both plant- and yeast-fed larvae reduce the proportion of mono-unsaturated PC and PS species at low temperatures, yeast-fed larvae replace them with di-unsaturated species, while plant-fed larvae increase species with 3, 4, and more double bonds (Figure 4). Thus, fatty acid unsaturation varies more strongly with temperature in plant-fed larvae than in yeast-fed larvae.

To confirm the dietary requirement for PUFAs, we analyzed the lipidomes of larvae raised on an equicaloric lipid-depleted food (+ a sterol addition) at either 14°C or 20°C. These animals pupariate at 20°C but fail to do so at 14°C, although they remain viable (Figure S3C). As expected, feeding with lipid-depleted food reduces levels of PUFA-containing lipids. Furthermore, larvae fed on lipid-depleted food do not increase the number of lipids containing PUFAs at low temperatures—just as on a yeast diet (Figure S3A).

While the total lipid fraction represented by sterols does not change with temperature on either diet, yeast-fed larvae increase the ratio of ergosterol to zymosterol as the temperature drops (Figure S4C). Oddly, this would be predicted to decrease, rather than increase, membrane fluidity at low temperature (Aresta-Branco et al., 2011; Hąc-Wydro et al., 2014).

In summary, although yeast-fed larvae do remodel their membrane lipids in response to temperature, these changes are insufficient to compensate membrane fluidity at low temperature—at least when protein-free liposomes are measured in the C-Laurdan assay. The key difference between plant and yeast-fed larvae appears to be that PUFAs are available only to plant-fed animals, enabling them to vary fatty acid unsaturation in membrane lipids over a broader range. This may explain why only plant-fed larvae are able to decrease membrane order and thus increase membrane fluidity at 12°C.

DISCUSSION

Here, we have shown that the common lab strain OregonR changes its feeding and egg-laying preference from yeast to plant material at temperatures below 15°C and that this shift extends the lower limit of the viable temperature range. Plant lipids enable development and survival at lower temperatures by contributing essential PUFAs needed to adjust membrane fluidity in the cold.

Whether *Drosophila melanogaster* membranes undergo homeoviscous adaption was previously unclear. Although some changes in lipid composition were observed at lower temperatures (Cooper et al., 2012; Cooper et al., 2014; Koštál et al., 2013; Ohtsu et al., 1998; Overgaard et al., 2006; Overgaard et al., 2008), membrane fluidity changes have not been measured in *Drosophila*. Our measurements show that *Drosophila melanogaster* do indeed adjust membrane fluidity to compensate for temperature, but only if they are allowed to feed on plant lipids and only when the temperature drops to 12°C. Fascinatingly, larvae raised at 12°C are able to alter their membranes such that they are more fluid at this temperature, without altering fluidity at higher temperatures. In the wild, larvae would be exposed to daily temperature fluctuations—the ability to construct membranes that resist low temperatures but are not compromised at higher temperatures could be useful in their normal environments.

Figure 3. Dietary Plant Lipids Decrease Membrane Order at Low Temperatures

(A) The percentage of larvae pupariating successfully at the indicated temperatures when fed lipid-depleted food supplemented with lipid extracts of yeast (red) or plant (black) food at the indicated temperatures. Error bars show standard error. Adjusted p values are indicated as follows: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. Only significant comparisons are represented.

(B–D) Panels show average generalized polarization (GP) values for C-larurdan emission measured at 12°C, 20°C, or 30°C from liposomes prepared from lipid extracts of larvae raised on yeast food (red) or plant food (black) at 12°C (B), 20°C (C), or 30°C (D). Lower GP values correspond to a higher membrane fluidity in liposomes. Averages are from three biological replicates. Error bars (gray) show standard deviation.

(E) The fraction of membrane lipids (without TAGs) represented by each major lipid class (in mol %) in larvae fed with plant (black) or yeast (red) food at the indicated temperatures is shown. Data show averages of three biological replicates, measured in two technical replicates. Bars indicate standard deviations.

(F) Principal-component analysis of membrane lipids (without TAGs) from plant- and yeast-fed larvae at four different temperatures. Black indicates samples from plant-fed larvae and red indicates samples from yeast-fed larvae. Samples from larvae raised at the same temperature are circled in different colors: blue (10°C), turquoise (13°C), orange (20°C), and red (30°C). The first principal component (PC1) describes diet-dependent differences and accounts for 52% of the variance. The second component (PC2) describes temperature-dependent differences and accounts for 16% of the variance.

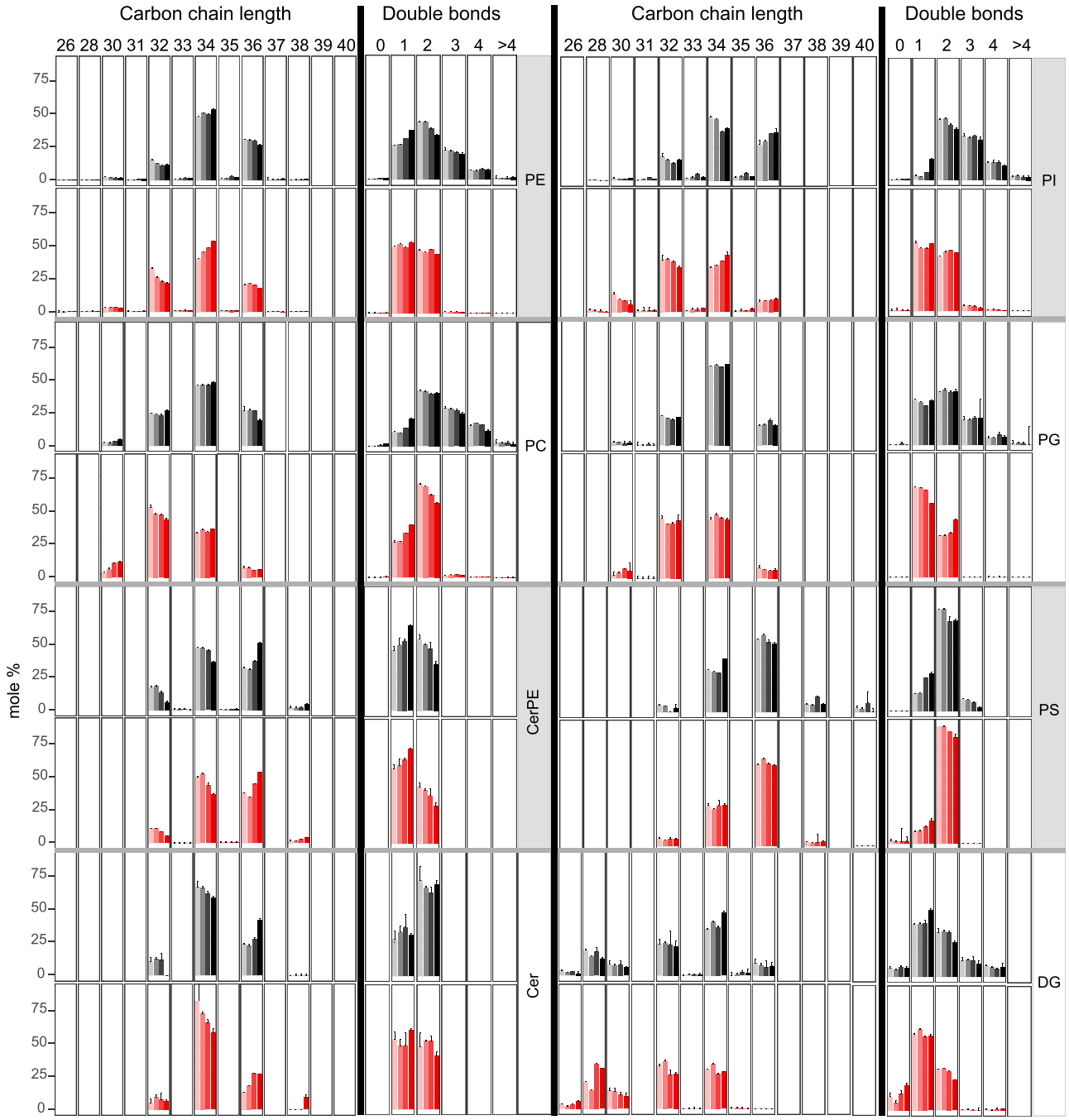

Figure 4. Effect of Temperature and Diet on Phospholipid Fatty Acid Chain Length and Unsaturation

The figure depicts the distribution of total fatty acid chain length and total number of double bonds, for species within each membrane lipid class, in lipidomes of plant- and yeast-fed larvae raised at different temperatures. Lipidomes from plant- and yeast-fed larvae are indicated by bars in shades of black and red, respectively. Increase in temperature is indicated by color saturation of the bars (very light, light, dark, and very dark represent 10°C, 13°C, 20°C, and 30°C, respectively). Fatty acid chain length and unsaturation profiles are shown as mole % within each class. Note that the number of double bonds in CerPE species includes the double bond in the sphingoid backbone. Data show averages of three biological replicates, measured in two technical replicates. Bars indicate standard deviations.

Although plant-fed larvae do alter membranes order to compensate for temperature, such compensation does not appear to be complete. When membrane order is measured at the temperature at which larvae are raised, liposomes from 12°C larvae are still more ordered than those of 20°C larvae, and those of 20°C larvae are more ordered than those of 30°C larvae (Figures 3B–3D, S2I, and S2J). Since larval metabolism and development run faster with temperature (Howe, 1967),

it may be that proportional changes in membrane fluidity help keep biological processes coordinated at different temperatures.

At temperatures above 12°C, plant- and yeast-fed animals produce membranes with indistinguishable properties in C-laurdan emission assays—despite their profoundly different lipid compositions. This suggests that at these more moderate temperatures, homeostatic mechanisms compensate for the different fatty acids and sterols present in the diet to maintain membrane biophysical properties. Similarly, mammalian tissue culture cells that are fed with PUFAs preserve membrane packing by increasing the synthesis of saturated lipids and cholesterol (Levental et al., 2018).

Our analysis shows that altered membrane properties of plant-fed larvae raised at low temperatures are caused by a combination of multiple lipidomic changes. One key important change is the increased incorporation of plant-derived PUFAs into membrane phospholipids at low temperatures. These fatty acids cannot be endogenously synthesized or obtained from yeast, explaining the requirement for plant food in survival at low temperature. Plant-fed larvae also store PUFAs in TAGs, which could provide a source of fatty acids for membrane remodeling when the temperature drops. *Drosophila* larvae can modulate other aspects of their lipid composition in response to temperature, independent of the diet they consume. Both plant- and yeast-fed larvae increase the PE/PC ratio and decrease the length and saturation of CerPE at low temperatures. However, these changes do not suffice to allow yeast-fed animals to modulate membrane fluidity in the cold. Furthermore, it is clear that large differences in fatty acid unsaturation alone do not necessarily change membrane order as measured by C-laurdan (see, for example, the very similar properties of membranes from plant- and yeast-fed larvae raised at 20°C). Our data suggest that a precise combination of these lipidomic changes tunes membrane properties in response to temperature. Thus, autonomously controlled changes in membrane lipid biosynthesis must be accompanied by a change in dietary lipids to effectively increase membrane fluidity at low temperatures. The unsaturated fatty acids provided by plants provide a key tool required to modulate membrane lipid unsaturation in the cold. Of course, cellular membranes *in vivo* comprise both lipid and protein components. It will be interesting to see whether changes in membrane proteins might also contribute to homeoviscous adaption *in vivo*.

Other studies of lab-reared *Drosophila melanogaster* have noted increases in the PE/PC ratio during different types of adult cold acclimation, but lipidomic analysis has generally revealed no or only minor changes in fatty acid unsaturation (Colinet et al., 2016; Koštál et al., 2011; MacMillan et al., 2009; Ohtsu et al., 1998; Overgaard et al., 2005, 2006; Overgaard et al., 2008), with one exception (Cooper et al., 2012). Our findings suggest that a variable content of plant-derived lipids in different laboratory food recipes might account for these inconsistent results. Interestingly, phytophagous insects collected in the wild at different times of the year do show seasonal changes in fatty acid unsaturation (Pruitt and Lu, 2008; Rozsypal et al., 2014; Vukašinović et al., 2015).

The changing attractiveness of yeast and plant olfactory cues at different temperatures allows *Drosophila* to alter their physiology and membrane properties by making a simple dietary choice. The olfactory receptors and neurons that detect specific yeast- and plant-derived compounds are well studied (Couto et al., 2005; Hallem and Carlson, 2006), as are the neurons and receptors responsible for hot and cold sensing. Like the olfactory neurons, many of the neurons that respond to temperature are located in the antennae (Gallio et al., 2011; Hamada et al., 2008; Liu et al., 2003). How these interface with each other to elicit temperature-dependent changes in food preference will be a key question for the future.

More broadly, our findings raise important questions about the ecology of wild *Drosophila* during different seasons. It will be interesting to explore whether they feed more on plants when the temperature drops and, if so, which ones. If wild populations of *Drosophila* do in fact shift between a diet of yeasts and plants at different temperatures, such a shift could help optimize their physiology for different seasons. Elevated insulin signaling caused by feeding on yeast in the summer would increase fertility and speed development to allow exploitation of a transient resource. Feeding and laying eggs on plant material as winter approaches would increase the success of larval development at low temperatures. It would also prepare emerging adults for overwintering, not only by preserving membrane fluidity but also by lowering insulin signaling to extend lifespans. Seasonal variations in diet and fertility also occur in mammalian populations, and it has been known for some time that plant and animal fatty acids have different effects on mammalian insulin signaling (Bhaswant et al., 2015; Oh et al., 2010; Yore et al., 2014). Our findings suggest that it may be productive to think about these differences in insulin signaling in the context of seasonal cycles.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and eight videos and can be found with this article online at https://doi.org/10.1016/j.devcel.2018.05.028.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.B., S.E., and U.C.; Investigation, M.B., T.G., A.S., O.K., E.P., B.B., A.P., and M.G.; Writing – Original Draft, M.B. and S.E.; Writing – Review & Editing, U.C., M.B., and S.E.; Funding Acquisition, A.S., U.C., M.B., and S.E.; Resources, U.C. and S.E.; Supervision, U.C., A.S., M.B., and S.E.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Coomasie G-250	SIGMA-Aldrich	CAS Number:6104-58-1
Ponceau S	SIGMA-Aldrich	CAS Number:6226-79-5
C-laurdan	Prof. B.R. Cho (South Korea)	N/A
lipid standards	Avanti Polar Lipids	N/A
Ergosterol	Sigma Aldrich	CAS Number:57-87-4
^{13}C uniformly labeled glucose	Euriso-top	CLM-1396
yeast nitrogen base	Difco	BDTM 291940
Deposited Data		
Original lipidomics datasets	Mendeley	https://data.mendeley.com/datasets/kmps9v9y6c/2
Experimental Models: Organisms/Strains		
W303 Y3358	MPI-CBG, Dresden	https://www.mpi-cbg.de/research-groups/current-groups/simon-alberti/research-focus
D.m.: *oregonR*	Bloomington (BDSC)	#5
Other		
Q Exactive instrument	Thermo Fischer	N/A
TriVersa NanoMate	Advion BioSciences	N/A
Fluoromax-4	Horiba Scientific (Unterhaching, Germany)	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Suzanne Eaton (eaton@mpi-cbg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster Stocks

OregonR (#5) and *or23b* mutant flies are available from Bloomington Stock Center.

Fly Stock Maintenance

If not indicated otherwise, flies were raised on BDSC Cornmeal Food (https://bdsc.indiana.edu/information/recipes/bloomfood.html) under a 12h light/12h dark cycle at 21-23°C.

Alternative Foods

The following ingredients are added into a total volume of 1 liter made up with water, brought to a boil and cooked for 10 minutes before pouring into vials as described (https://bdsc.indiana.edu/information/recipes/bloomfood.html).

Yeast Food

Yeast extract: 20g, Peptone (Soy): 20g; Sucrose 30g; Glucose: 20g; Brewers yeast 80g

Plant Food

Sugar beet: 30g; Malt: 45g; Sunflower-Oil: 2g; Peptone (Soy): 20g; Cornmeal: 55g; Glucose: 75g

Lipid Depleted Food

Yeast extract (Sigma 73145): 100g; Glucose: 100g

Methylparaben and Propionic acid are added to each food (see Bloomington stock center: https://bdsc.indiana.edu/information/recipes/bloomfood.html).

METHOD DETAILS

Behavioral Assays

Egg Laying Assay

10d after eclosion, 40 mated females and 20 males were transferred onto conditioned food choice assay plates and shifted to the specified conditions. Assay-plates were collected after 12h (30 and 20°C), 48h (15°C) and 72h (12°C), divided into the sectors shown in Figure 1A. Food-containing sectors were manually assayed for embryo numbers.

Food Choice Assay (Adults)

Yeast-pellet (K classic yeast, Kaufland) were suspended in 5ml 10% Glucose, incubated for 30' at RT, stained with Coomasie blue G-250 (Sigma-Aldrich) and then stored at 3°C (>24h). Plant food paste was stained with Ponceau S (Sigma-Aldrich) and stored at 3°C (>24h). Food types were placed in defined rectangular areas (5x10mm) on opposite sides of an agar plate 40mm distant from the plate center (1% Agar, Greiner, circular 100x15mm).

Flies were raised at 21-23°C on normal food and eclosed virgin animals transferred onto YF. 10d after eclosion, 20 mated females and 10 males were transferred onto conditioned assay plates, and kept at the indicated temperatures for 24h or 72h. Collected flies were photographed and visually assayed for gut color as an indication of food preference.

Food Choice Assay (Larvae)

Embryos were collected at 1h intervals at 21-23°C, aged for 24h, and hatched 1st instar larva were transferred onto preconditioned assay plates (see food choice assay (adults)). Samples were manually assayed for gut color after 72h (exception: larva kept at 12°C or 8°C were sampled after 216h).

Motility Assay

Flies were raised on PF or YF at 21-23°C transferred at 12d after eclosion to the specified temperatures for 72h. Thereafter, motility and coordination were video-recorded in temperature controlled assay chambers. In brief, the motility assay chamber consists of a 3cm tube (d=1cm) connected to a petri-dish (d=6cm). Flies were transferred into the tube und shifted for 15min to 8°C. After vortex at max speed for 5 sec, the assay platform was transferred into a water bath set to the specified temperature and video-monitored. In the assay plate, flies are forced to climb up the tube and then are allowed to explore the planar plate area.

Survival Assays

Pupariation Assay. 4h embryo collections were washed with distilled water, transferred onto fresh apple-juice agar plates and aged for 5-6h at 21-23°C, then transferred onto specific foods (20embryos/vial) and raised at different temperatures. Each vial was visually assayed for pupariation at 24h intervals.

Lifespan Assay. Flies were raised on normal food at 21-23°C or 15°C (cold adapted flies) and eclosed animals transferred onto YF or PF. After 10d, mated females (20) and males (10) were transferred onto the respective freshly cooled (15°C) food vials and kept at this temperature for 48h. Subsequently vials were shifted to indicated breeding conditions. Surviving females were counted at 72h intervals and transferred onto fresh food if required.

Lipid Rescue Assay. 4h embryo collections were washed with distilled water, transferred onto fresh apple-juice agar plates and aged for 5-6h at 21-23°C, then transferred onto specific foods (20embryos/vial) and raised at different temperatures conditions. The success of larval development was monitored by counting the number that pupariated.

Biochemistry

Lipid Extraction

For each lipid sample, 30 feeding 3rd instar wild type larvae (between 100-110 hrs after egg collection at 25°C) raised under the specified conditions were dissected (on ice in HEPES-buffered saline (HBS): 25mM HEPES, 150mM NaCl, pH 7.25) to remove the gut and CNS, snap-frozen in liquid nitrogen and stored at -80°C until lipid extraction. Tissue samples were thawed on ice, homogenized in HBS using an IKA® ULTRA-TURRAX® disperser (level 5, 1min), and lipid-extracted by the BUME method. Extracted lipids were stored in chloroform / methanol (2:1) solution at -80°C.

Liposome Preparation

A volume containing 40 nmol phospholipids was dried under a stream of pressured air and left under vacuum for 2 h to ensure complete removal of solvents. The lipid film was rehydrated with 200 µl HBS with vigorous shaking for 30min, followed by 10 freeze-thaw cycles, and extruded through a 100nm filter to obtain homogeneous 100nm unilamellar liposomes.

Fluorescence Spectroscopy

Membrane order was assessed by determining C-laurdan generalized polarization (GP) indices of liposomes prepared from fly lipids. Liposomes (200µM phospholipids) were stained with C-laurdan (0.4µM) and incubated for 30min in the dark at room temperature. Fluorescence spectra were obtained with a FluoroMax-4 spectrofluorometer (Horiba) equipped with a temperature-controlled Peltier element (Newport) at 12, 20, and 30°C. Excitation was 385nm and emission was recorded at 400-600nm with 1nm. Spectra were background-corrected and GP values were calculated as described in Kaiser et al. (2009) (#235).

Mass Spectroscopy

Standards for Lipid Quantification. Synthetic lipid standards and Ergosterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA), Sigma Aldrich (Steinheim, DE) and Toronto Research Chemicals (Toronto, CA). 99% isotopically pure ^{13}C uniformly labeled glucose was purchased from Euriso-top (Saint Aubin, FR) and yeast nitrogen base without amino acids from Difco (LE Pont de Claix, FR). All used solvents were of at least HPLC grade. Stocks of internal standards were stored in glass ampoules at -20°C until used for the preparation of internal standard mix in 10:3 MTBE/MeOH. 700 µl internal standard mix contained: 445 pmol Cholesterol D_7, 259 pmol Zymosterol D_5, 248 pmol Campesterol D_6, 239 pmol Sitosterol D_6, 155 pmol Lanosterol D_6, 418 pmol Stigmasterol D_6, 164 pmol ^{13}C Ergosterol, 208 pmol 50:0 TG D_5, 58 pmol 34:0 DG D_5, 138 pmol 25:0 PC, 87 pmol 13:0 LPC, 54 pmol 25:0 PS, 147 pmol 25:0 PE, 43 pmol 13:0 LPE, 96 pmol 25:0 PI, 55 pmol 25:0 PG, 73 pmol 30:1 Cer, 62 pmol 25:0 PA, 45 pmol 13:0 LPA, 148 pmol 29:1 CerPE, 32 pmol 13:0 LPI.

^{13}C uniformly labeled Ergosterol was produced in the prototrophic yeast strain W303 Y3358. A single colony was inoculated in 25 ml sterile filtered synthetic defined medium (20 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids). Y3358 was incubated for 21 h at 30°C to reach stationary phase and then pelleted by centrifugation (10 min, 4000 g). The medium was removed and the pellet was washed twice with 1 ml H_2O. The corresponding pellet was split into 6 samples. These pellets were reconstituted in 300 µl isopropanol (IPA) each and homogenized with 0.5 mm zirconia beads on a tissuelyser II for 20 min at 30 Hz. The dried homogenates were saponified with 1 ml 3% KOH in methanol for 2 h at 90°C each. ^{13}C Ergosterol was extracted twice with 2 ml hexane and 1 ml H_2O and the combined organic phases from all samples were evaporated. Finally, ^{13}C Ergosterol was reconstituted in 1 ml MeOH and stored at -20 C. ^{13}C Ergosterol was quantified by commercially available ^{12}C Ergosterol and performing parallel reaction monitoring (for exact settings see below).

Lipid Extraction and Quantification by Shotgun Mass Spectrometry. 5 *Drosophila* larvae were homogenized with 1 mm zirconia beads in a cooled tissuelyzer for 2 x 5 min at 30 Hz in 500 µl IPA. An aliquot corresponding to 1 larvae was evaporated in a vacuum desiccator to complete dryness. Lipid extraction was performed according to (Sales et al., 2016). In brief, 700 µl internal standard mix in 10:3 MTBE/MeOH was added to each sample and vortexed for 1 hat 4°C. After addition of 140 µl H_2O samples were vortexed for another 15 min. Phase separation was induced by centrifugation at 13400 rpm for 15 min. The organic phase was transferred to a glass vial and evaporated. Samples were reconstituted in 300 µl 1:2 MeOH/$CHCl_3$. 100 µl were transferred to a new vial and used for lipidome analysis. To quantify sterols 200 µl of lipid extract were evaporated and acetylated with 300 µl 2:1 $CHCl_3$/acetyl chloride for 1 h at room temperature. After evaporation sterol samples were reconstituted in 200 µl 1:2 MeOH/$CHCl_3$. For sterol measurements 15 µl of sample were diluted with 85 µl 4:2:1 IPA/MeOH/CHCl3 + 7.5 mM ammonium formate. For lipidome and PS measurements 5 µl of sample were diluted with 95 µl 4:2:1 IPA/MeOH/CHCl3 + 7.5 mM ammonium formate and 4:1 EtOH/$CHCl_3$ + 0.1% trimethylamine, respectively.

Mass spectrometric analysis was performed on a Q Exactive instrument (Thermo Fischer Scientific, Bremen, DE) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, USA) using nanoelectrospray chips with a diameter of 4.1 um. The ion source was controlled by the Chipsoft 8.3.1 software (Advion BioSciences). Ionization voltage was + 0.96 kV in positive and − 0.96 kV in negative mode; back pressure was set at 1.25 psi in both modes. Samples were analyzed by polarity switching (Schuhmann et al., 2012). The temperature of the ion transfer capillary was 200 °C; S-lens RF level was set to 50%. For lipidome measurements, each sample was analyzed for 3.3 min. FT-MS spectra were acquired within the range of m/z 400–1000 from 0 min to 1.5 min in positive and within the range of m/z 350–1000 from 1.7 min to 3.3 min in negative mode at a mass resolution of R m/z 200 = 140000, automated gain control (AGC) of 3 x 10^6 and with an maximal injection time of 3000 ms. Acetylated sterols were quantified by parallel reaction monitoring (PRM) FT-MS/MS in an additional measurement. FT-MS spectra within the range of m/z 350-1000 were acquired from 0 min to 1.5 min and PRM were acquired from 1.5 min to 5 min. For FT-MS/MS micro scans were set to 1, isolation window to 0.8 Da, normalized collision energy to 12.5%, AGC to 5 × 10^4 and maximum injection time to 3000 ms. PS was additionally measured for 1.5 min in neg FT-MS mode with the same parameters as mentioned above. All acquired spectra were filtered by PeakStrainer (https://git.mpi-cbg.de/labShevchenko/PeakStrainer/wikis/home) (Schuhmann et al., 2017). Lipids were identified by LipidXplorer software (Herzog et al., 2012). Molecular Fragmentation Query Language (MFQL) queries were compiled for acetylated sterols, PC, PCO, LPC, LPCO, PE, PEO, LPE, PI, LPI, CerPE, Cer, PA, PG, TG, DG lipid classes. The identification relied on accurately determined intact lipid masses (mass accuracy better than 5 ppm) and signal to noise threshold higher than 3. Lipids were quantified by comparing the isotopically corrected abundances of their molecular ions with the abundances of internal standards of the same lipid class. For acetylated sterols, the specific fragment (loss of acetyl group) was used for quantification.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of the Lipidomics Data

Lipidomes were explored by means of principal component analysis (PCA) (Jolliffe, 2002) using the R package "factoextra" (Kassambara and Mundt, 2015) after transforming the data to mole percent. Lipidomics data represent an average of three biological replicates, each measured in three technical replicates. Error bars indicate standard deviations.

Statistical Tests

Data collected from egg laying, food choice and pupariation assays were tested for significant differences using Anova and Tukey procedure as a post-hoc test in R.

DATA AND SOFTWARE AVAILABILITY

The accession number for the original lipidomics data reported in this paper is [Mendeley]: https://data.mendeley.com/datasets/kmps9v9y6c/2.