Perceptive costs of reproduction drive ageing and physiology in male *Drosophila*

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Costs of reproduction are thought to result from natural selection optimizing organismal fitness within putative physiological constraints. Phenotypic and population genetic studies of reproductive costs are plentiful across taxa, but an understanding of their mechanistic basis would provide important insight into the diversity in life-history traits, including reproductive effort and ageing. Here, we dissect the causes and consequences of specific costs of reproduction in male *Drosophila melanogaster*. We find that key survival and physiological costs of reproduction arise from perception of the opposite sex, and they are reversed by the act of mating. In the absence of pheromone perception, males are free from reproductive costs on longevity, stress resistance and fat storage. The costs of perception and the benefits of mating are both mediated by evolutionarily conserved neuropeptidergic signalling molecules, as well as the transcription factor dFoxo. These results provide a molecular framework in which certain costs of reproduction arise as a result of self-imposed ‘decisions’ in response to perceptive neural circuits, which then orchestrate the control of life-history traits independently of physical or energetic effects associated with mating itself.

Costs of reproduction represent a set of putative fitness trade-offs through which enhanced reproductive effort compromises future survival and limits lifespan. Putative costs of reproduction have been characterized as either ecological or physiological in nature\(^1\), and they have been described in nearly all taxa, from brine shrimp\(^2\), nematodes\(^3\) and fruit flies\(^4\) to rodents\(^5\) and primates\(^6\), including humans\(^7,8\). Ecological costs of reproduction represent environmental dangers that result from reproductive behaviour, including increased risk of predation and disease. As such, their causes and consequences are often identifiable, and best studied in natural conditions\(^8\). In human societies and in laboratory settings, ecological costs of reproduction are reduced or largely eliminated.

Physiological costs of reproduction manifest because limited resources necessitate that most biological functions receive allotments that are inadequate to sustain their long-term function, leading to a reduction in future health and survival. They also involve physiological decisions through which a finite amount of available energy is partitioned between reproduction and survival such that neither is at its theoretical maximum\(^10\). Physiological costs of reproduction have been described at the phenotypic and population genetic levels in a wide range of species, and they are generally recognized as the most significant components underlying life-history trade-offs\(^9\). The biological mechanisms underlying these costs, however, have been decidedly more difficult to identify\(^1,10-12\).

Mechanistic insights about costs of reproduction have come largely from experiments using the fruit fly *Drosophila melanogaster\(^11-17\)* and are reviewed in ref.\(^12\). These experiments have examined costs in both males and females, and surprisingly, they have failed to implicate physiological or energetic trade-offs. Even studies in which a connection to energetics has been shown through dietary restriction\(^14\) or by measuring energy stores\(^19\) conclude there is no simple trade-off between reproduction and somatic investment. In female flies, costs of reproduction arose in part from the transfer of the seminal fluid protein sex peptide from males to females during mating, which increased female mortality rates and decreased lifetime reproductive output. This occurred despite an increase in food intake\(^20\) and decrease in mating frequency\(^19\), both of which should result in a net increase in energy availability. In males, the molecular mechanisms underlying reproductive costs are a mystery. One study demonstrated that male flies exposed to cauterized females, which do not mate, suffered from decreased lifespan beyond those of males exposed to normal females, revealing a cost of reproduction that manifests in the absence of mating itself\(^22\). The authors suggested increased energy expended by courtship as the cause for this effect, but this seems unlikely, as recent studies have shown that activity in general and exercise in particular are not sufficient to affect lifespan\(^21\).

Here, we dissect the mechanisms underlying the costs of reproduction on both longevity and specific markers of health in male *Drosophila*. We show that changes in survival and key physiological costs occur in response to the animal’s perceived reproductive environment, independent of any physical or energetic effects associated with mating itself. We also find that mating promotes health and lifespan in this context by reversing the phenotypic consequences of perception, and that this phenomenon is potentiated by the neuropeptide coronazin (Crz), which is the fly homologue of vertebrate gonadotropin-releasing hormone (GnRH). In the absence of perception, male *Drosophila* experience no measurable survival costs of mating itself. Physiological changes in peripheral tissues that occur in response to perceptive perception require the activity of the transcription factor dFoxo, independent of systemic insulin signalling. Self-imposed biological responses to reproductive perception are likely to exist across taxa\(^23\) and therefore may constitute
Results
Pheromone perception drives costs of reproduction and mating ameliorates them. Recent results from experiments using Drosophila and the nematode Caenorhabditis elegans suggest that costs of reproduction on lifespan may be biologically regulated responses to the perception of the opposite sex, rather than physical constraints or energetic limitations in reproductively active animals23–25. Male Drosophila that perceived female pheromones, but did not mate, showed decreased lifespan, triglyceride stores (a major storage form for lipids in flies) and stress resistance26. Similar effects of sexual perception were observed in hermaphrodite C. elegans, suggesting that this phenomenon may be evolutionarily conserved27.

To explore the role of pheromone perception in costs of reproduction on health and longevity in male Drosophila, we manipulated the perceived sexual environment of experimental animals independently of their mating opportunities. To do this, we replaced the pheromone profile normally produced by a male or female fly with one characteristic of the opposite sex. Male flies that produced female pheromones (termed ‘feminized males’ and symbolized by $\delta^f$) were created by targeting expression of the sex determination gene, *tra*, to the oenocytes (via OK72-Gal4), whereas masculinization of female flies ($\delta^m$) was accomplished by expressing tra-RNA interference in a similar way28. These transformed animals, together with animals that produced pheromones consistent with their genetic sex (noted as $\delta^f$ and $\delta^m$ for males and females, respectively), constituted four groups of ‘donor’ animals that were independently housed with experimental males (Fig. 1a).

We found that phenotypic effects on survival and metabolism were due to sexual perception. When we housed experimental males with feminized male donor animals ($\delta^f$), thus precluding the possibility of mating, the presence of female pheromones significantly decreased starvation resistance, reduced total levels of stored triacylglyceride (TAG) and shortened lifespan compared with experimental males housed in the presence of male donors ($\delta^m$ versus $\delta^f$; Fig. 1b–d; Supplementary Fig. 1), as previously shown23. However, we failed to observe significant differences in these phenotypes when comparing experimental males that were housed with masculinized female donor flies, which produced male pheromones, to those housed with male donor flies ($\delta^f$ versus $\delta^m$).

Our failure to observe measurable costs of reproduction on survival, fat storage, or stress resistance was not because males did not mate with masculinized females. In competitive environments, males only modestly preferred females with female pheromone profiles to those that had been masculinized (27/41 = 66% of the time, $P = 0.06$, binomial exact test). The majority of flies (31/41 = 76%) mated with both females, and males that first chose a $\delta^f$ female were slower to remate than males that first chose a $\delta^m$ female (Supplementary Fig. 2a). Furthermore, in experimental conditions where males were exposed to only one type of female, we routinely observed similar numbers of fertilized eggs in cohorts exposed to $\delta^f$ and $\delta^m$ donor females, and mating latency (Supplementary Fig. 2b) and fertilization rates (Supplementary Fig. 2c) were not significantly different.

Exposure to female pheromones reduced TAG storage, stress resistance and lifespan. However, when we compared the effects of female pheromones as presented by male ($\delta^f$) or female ($\delta^m$) donor animals, we found that the negative effects of female pheromone exposure were significantly ameliorated when experimental males were exposed to $\delta^f$ females and thus allowed to mate (compare red with yellow groups in Fig. 1b–d; Supplementary Fig. 1). This is unexpected, considering that costs of reproduction are thought to represent energetic requirements that include courtship and copulation.

To investigate whether aggressive or courtship behaviours were responsible for the phenotypic differences we observed, we collected video of freely behaving experimental flies in each of the four exposure conditions. We did not observe any appreciable frequency of aggression (for example, male–male escalation) in any treatment, confirming results reported previously23. We also quantified reproductive interactions (that is, courtship behaviours)

![Figure 1 | Pheromone exposure, and not mating, drives the physiological costs of reproduction.](https://example.com/figure1.png)
Pheromones and mating have distinct effects on the neurometabolome. Owing to the involvement of sensory circuits in the costs of reproduction on survival, we predicted that short-term effects of perception would influence the brain states of experimental males. We therefore compared metabolite abundances in heads of experimental males that had been exposed to $\varphi^{(d)}$, $\varphi^{(f)}$, or $\varphi^{(v)}$ donor animals for 48 h. Untargeted metabolite analysis identified 524 and 409 metabolites present in all treatments for positive and negative modes, respectively (see Supplementary Information for raw data). Using a randomization procedure together with principal component analysis (PCA), we identified a single principal component (PC5) that significantly distinguished different treatments (Fig. 2a,b). Strikingly, this principal component separated groups based only on pheromone exposure; the neurometabolome of male flies exposed to $\varphi^{(d)}$ (pheromones alone) essentially overlapped in this space with the neurometabolome of male flies exposed to $\varphi^{(v)}$ (pheromones and mating). Both were clearly distinct from that collected from males exposed to $\varphi^{(f)}$ donor animals. These results suggest that the neurological signature of reproductive activity is faithfully recapitulated by the mere perception of available females.

We next asked whether mating influenced the effects of perception on the neurometabolome. We therefore repeated the previous experiment with the addition of a fourth exposure treatment in which experimental males were exposed to masculinized females ($Q^{(d)}$). In this case, we identified two positive mode metabolome principal components (PC2 and PC3) that provided a statistically significant ability to resolve treatments (Fig. 2c). One principal component (in this case PC3) separated the neurometabolomes of experimental males according to pheromone perception, grouping $\varphi^{(d)}$ and $Q^{(d)}$ exposure treatments together, and separating them from $\varphi^{(v)}$ and $\varphi^{(f)}$ exposures (Fig. 2d; Supplementary Fig. 5a). PC2 separated the treatments according to mating status, grouping the female exposures apart from the male exposures (Fig. 2d; Supplementary Fig. 5a). Both PC2 and PC3 provided significant separation of their respective phenotypes when compared with random permutations (Supplementary Fig. 5a). Similar results were observed using the negative mode data (Supplementary Fig. 5b). PC2 and PC3 showed increased weighting of molecules that were differentially produced between either mating/non-mating groups or male/female pheromone groups, respectively, reinforcing the importance of these components (Supplementary Fig. 5c). As PC2 and PC3 are orthogonal, these data indicate that the impact of mating on the neurometabolome is distinct from that of pheromone perception, suggesting that mating does not strictly reverse the effects of perception but instead induces distinct changes that ameliorate its consequences.

The top 20 metabolites based on PC2 or PC3 loadings are listed in Supplementary Table 1 (see Supplementary File 1 for all metabolite data). Metabolites associated with pheromone exposure were

Figure 2 | Pheromone exposure and mating drive separate global changes in the neurometabolome. a, Observed (blue) and randomized (grey) distributions of the ability of individual principal components to effectively distinguish neurometabolic signatures of experimental males. No statistical difference was observed between these two distributions ($P = 0.95$, Kolmogorov–Smirnov test). This analysis identified a single principal component, PC5, that provides significant separation between groups of experimental males ($P = 0.017$, permutation test, see Methods). b, Principal component plot showing the distribution of samples for each treatment. N = 5 biological replicates of 40–50 fly heads per group. c, Observed (blue) and randomized (grey) distributions of the ability of individual principal components to effectively distinguish neurometabolic signatures of experimental males. No statistical difference was observed between these two distributions ($P = 0.44$, Kolmogorov–Smirnov test). This analysis, which included animals exposed to all four types of donor fly, identified two PCs (PC2 and PC3) that exhibited statistically significant ability to distinguish exposure groups ($P = 0.0018$ for PC2, $P = 0.0056$ for PC3, permutation test, see Methods). d, Principal component plot showing the distribution of samples for each treatment. Neur metabolites in PC2 distinguish exposures based on mating status, whereas neur metabolites in PC3 distinguish exposures based on pheromone perception. Analyses represent mass spectrometry analysis of metabolites identified under positive mode. N = 5 biological replicates of 40–50 fly heads per group.

in male flies housed in each of our four treatments. Males exposed to feminized males exhibited an equal reproductive investment (for example, courtship time) with donor animals as did experimental males exposed to females, although these interactions were shorter on average (Supplementary Fig. 2d,e). Less time was devoted to masculinized females and control males. These results were supported by a wing-damage assay over a longer (14 day) time period, in which minor wing damage was more common in males exposed to female pheromones than male pheromones, independent of the donor animal’s sex (Supplementary Fig. 2f). We therefore conclude that survival differences among our four exposure treatments are not driven by different levels of aggression, and they do not correlate well with levels or intensity of courtship.

To further test the hypothesis that the male’s perception of female pheromones is required for the costs of reproduction on health and longevity, we repeated our survival experiments using experimental males that carried a loss of function mutation in the pheromone receptor ppk23 (ref. 24). Unlike control males (Supplementary Fig. 3a), ppk23 mutant males showed no lifespan differences whether they were exposed to $\varphi^{(d)}$ (no mating or pheromones), $Q^{(d)}$ (mating alone), or $Q^{(v)}$ (pheromones and mating) donor animals (Fig. 1e). ppk23 mutant males were therefore long-lived in a mixed-sex environment when compared with wild-type males (Supplementary Fig. 3b). In the absence of female pheromones, the ppk23 mutation had no influence on lifespan (Supplementary Fig. 3c). Loss of ppk23 also prevented the cost of reproduction effects on starvation resistance (Supplementary Fig. 3d,e). In mixed-sex environments, reproductive output from ppk23 males (as measured by the number of offspring sired per vial) was surprisingly higher than that of control males throughout life, despite their extended lifespan (Supplementary Fig. 4). One possible explanation is that mating with the ppk23 mutant males is less harmful to females, allowing for increased reproductive output. Together, these data indicate that the lifespan extension in ppk23 mutant males results from the elimination of perceptive costs of reproduction on survival, and that reproduction itself is not costly.
We next sought to identify those that mediate the effects of perceptive costs of reproduction on starvation resistance and TAG storage (Supplementary Table 2). This work identified the transcription factor dFoxo as a regulator of these effects. Indeed, two distinct dFoxo mutant alleles were resistant to pheromone effects on starvation resistance and TAG abundance (dFoxo<sup>24</sup> (ref. 28) and dFoxo<sup>W24</sup> (ref. 29); Fig. 4a,b and Supplementary Fig. 7a,b, respectively), as were flies that were trans-heterozygous for the two alleles (Supplementary Fig. 7c). Furthermore, the dFoxo<sup>W24</sup> mutation nearly eliminated the effect of pheromones on lifespan (Fig. 4c), and the dFoxo<sup>W24</sup> mutation significantly reduced it (Supplementary Fig. 7d). Pheromone exposure also decreased the expression of the dFoxo target gene 4EBP/Thor (ref. 29) (Supplementary Fig. 7e), suggesting a reduction of dFOXO activity upon pheromone exposure.

In Drosophila, three of the eight insulin-like peptides (dilp2, dilp3, and dilp5) are produced in specialized neurosecretory cells in the pars intercerebralis (PI), the fly analogue of the vertebrate hypothalamus 31. Previous studies have suggested that the PI, and particularly dilp2, modulate lifespan in flies 32,33, likely by inactivating dFOXO. Surprisingly, pheromone exposure did not alter the abundance of circulating dILP2 in the haemolymph (Fig. 4d), and simultaneous loss of the three centrally produced insulin-like peptides did not prevent the effects of pheromone exposure on starvation resistance or lifespan (Fig. 4e,f). Moreover, manipulation of both target of rapamycin (TOR) signalling and sir2 gene function did not prevent the effects of pheromone perception on lifespan (Supplementary Fig. 8), suggesting that the effect of pheromone exposure on ageing does not require other dFOXO-related ageing pathways.

**Discussion**

Costs of reproduction in male *D. melanogaster* have been reported to compromise health and limit lifespan 32. By taking advantage of genetic reagents that allowed us to manipulate mating opportunities independently of the social environment, we were able to investigate the mechanisms underlying these costs. In the absence of female pheromones or the ability to perceive them, reproductive behaviours, and in particular mating itself, have no negative effects on several major life-history traits in males, including stress resistance, fat storage and lifespan. Surprisingly, the act of mating itself was found to be beneficial when males perceived pheromones. These reproductive costs are, therefore, not inevitable but instead are self-imposed in nature.

The neuropeptides NPF and corazonin mediate self-imposed costs of reproduction. Our neurometabolomic data suggested that mating and pheromone exposure have distinct effects on brain state, indicating that mating may act through parallel neural circuits to rescue the deleterious consequences of pheromone exposure. We therefore sought to identify distinct subsets of neurons that are required for the full effect of pheromones or mating. *Drosophila* neuropeptide F is the fly homologue of vertebrate neuropeptide Y, and it has been implicated in feeding and reward behaviours 34,35. The authors of a previous study 36, inhibited npf-expressing neurons by expressing a temperature-sensitive dynamin mutant shibire (shib> mutants) and found that this significantly reduced the effects of female pheromones on male stress resistance. We reproduced those findings and implicated NP-F directly using an npf mutant allele. We found that npf is required for the full effect of female pheromones on male lifespan (Fig. 3a), establishing that it is a key neuropeptide involved in modulating the perceptive costs of reproduction on longevity.

In a candidate screen designed to reveal suppressors of pheromone effects, we found that neurons expressing the neuropeptide corazonin, which is the fly homologue of vertebrate GnRH, mediate the beneficial effects of mating. When we inhibited crz-expressing neurons in adult experimental males, mating no longer rescued the deleterious effects of pheromone perception on lifespan (Fig. 3b). When we activated crz-expressing neurons, however, mating completely reversed pheromone costs, such that mating and exposure to female flies had no effect on male lifespan (Fig. 3c; Supplementary Fig. 6). Notably, activating crz-expressing neurons was not sufficient to increase lifespan in same-sex cohorts, suggesting that this peptide specifically mediates the effect of mating on lifespan (Fig. 3c).

The transcription factor dFOXO modulates the physiological effects of perceptive costs of reproduction. We next sought to identify signalling pathways in peripheral tissues that are responsible for the effects of pheromone perception. Based on gene expression studies of ageing and pheromone effects in flies and *C. elegans* 37–41, we carried out a survey of candidate genes and interventions to enrich glycerophospholipid metabolism (P = 0.022, Fisher’s exact test), which plays an important role in modulating neural activity. metabolites associated with PC2, the mating axis, were not significantly enriched for any specific pathway.
neurons reach npf-expressing neurons, as the two populations do not seem to be directly connected. Similarly, determining whether and how neuronal NPF and CRZ signalling influences dFOXO activity in peripheral tissues would provide important insight into the cell non-autonomous nature of reproductive physiology. Surprisingly, several pathways that interact with FOXO to modulate ageing (dILPs 2, 3 and 5, along with TOR and sirtuin signalling) are dispensable for pheromonal effects on lifespan, suggesting the possibility of undiscovered regulatory mechanisms.

Several lines of evidence suggest that the signalling processes we describe may influence costs of reproduction in female flies and in individuals of other species. Matting reversed observed costs of courtship that female Drosophila may have experienced on exposure to males, and once the deleterious effects of sex peptide were removed, females were largely free of mating costs. Furthermore, the influential pathways that we characterized in male Drosophila are evolutionarily conserved. The vertebrate homologue of corazonin is GnRH, which is known to regulate sexual behaviour in addition to reproductive physiology. GnRH-producing neurons are anatomically altered by social interactions in cichlid fish, and GnRH itself has been linked to mammalian ageing. Notably, NPY, the vertebrate homologue of npf, interacts with GnRH neurons in mammalian systems. These results suggest the NPY–GnRH axis deserves attention as a mechanism that might influence healthy ageing in humans and could account in part for variation in life-history strategies across species in nature.

The notion that some, if not all, costs associated with reproductive effort result from the balance between decisions in response to reproductive opportunity, and the outcome or reward of those actions, provides new context to existing literature and suggests new avenues for future research. Previous work demonstrated that males exposed to cauterized females live shorter than males exposed to control females. This finding was interpreted to be a result of energy expenditure of courtship and was termed a ‘cost of courtship’. In light of our findings, these results might now be interpreted as owing to pheromone exposure, with males exposed to non-cauterized females having the negative effects of pheromone exposure partially rescued by the beneficial effects of mating. In this view, costs of courtship result not from courting itself but instead from the drive to court, and the magnitude of the cost is influenced by whether male flies achieve reproductive success. This perspective may invoke reflections on human emotions, including frustration and desire, which may share mechanistic building blocks, or ‘emotion primitives’ that are conserved across species. In humans, psychological factors, such as perceived quality of life, have been linked to protective effects on mental health in advanced age, and copulation has been associated with a variety of health benefits. Thus, future research on reproductive costs and ageing may benefit from a focus on central neurological states and their influence on life-history traits through orchestrated physiologic responses in peripheral tissues.

Methods

General fly husbandry. All flies used in this study (including experimental and donor animals) were raised using the same methods. Eggs were collected from yeasted grape juice agar plates, and 32 μl of eggs were placed onto bottles containing a modified Caltech medium (CT), a commonly used cornmeal-based food. Unless otherwise noted (for example, for neuronal activation/inhibition experiments), all flies were kept in a controlled humidity incubator at 25 °C in 12 h light:12 h dark conditions. Flies were collected into bottles containing 10% yeast/sucrose food within 24 h of emergence, and (unless specified as virgin) were allowed to mate for 2–3 d, after which they were sexed into groups of 25 in vials containing 10% yeast/sucrose food. Flies were maintained on 10% yeast/sucrose food for the remainder of their life, with the exception of the candidate survey, during which flies were placed on 30% sucrose/5% yeast food for 4 d prior to being sacrificed for physiologic assessments.

Fly stocks. The standard laboratory stocks yw, w1118 and Canton-S were originally obtained from the Bloomington Stock Center. The OK72-Gal4 line, UAS-irak2.1 line, UAS-Gal80 line and fox2 mutant lines were also obtained from the
nullification. npf mutants were created by the transgenic CRISPR/Cas9 technique as previously described. The gene-specific 20 bp sequence of the guide RNA was GCCCTTGCCCTCCTTACGGCC. The deletion is an 11 bp deletion near the 3’ end of the coding region and is as shown below:

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WT GGTTGCGGTGGTGCCGTTTCCCCCTTGCGGGGC
GTCGGTGCCGAG
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52K GGTTGCGGTGGTGCCGTTTCCCCCTTGCGGGGC
GTCGGTGCCGAG
GCGAGTGGAGGAGGTCGCACTC

Production of donor flies. Male donor flies were produced by crossing either w1118; UAS-TRA+; virgin females or w;UAS-TRA+; virgin females to either w1118; OK72-Gal4; males to create dGal4 donor flies or to w1118;UAS-TRA+; genetic controls (to create dGal4 donor flies). Female donor flies were produced similarly, except w;UAS-TRA+; virgin females were crossed to w1118;OK72-Gal4;+ males to generate dGal4 donor flies and to w1118;+/+; + genetic controls to create dGal4 donor flies.

Exposure to donor flies. Experimental flies were exposed to donor animals in a ratio of 5:25 experimental to donor flies, unless otherwise stated (see also ref. 7). For physiologic and stress resistance assays, exposure began 8–10 d after eclosion, except when performing the screen, when 12–14 d old flies were used to allow for time on 30% sucrose/5% yeast food. For these experiments, experimental flies were exposed to donor animals for 48 h, after which donor flies were removed and experimental flies were assayed. For lifespan experiments, experimental flies were exposed to donor animals beginning on day 2 following eclosion (after sexes had been separated), and exposure continued over the lifetime of the flies.

Lifespan assays. After beginning exposure, lifespan data were collected using Dille computer software. For each lifespan, 100 experimental flies (2 replicate vials each containing 5 experimental and 25 donor flies) were established for all treatment/genotype groups. Flies were transferred to fresh vials and food every 2–3 d, at which time deaths were recorded. Donor flies were repleted when approximately 75% remained (~28 d at 25°C, ~21 d at 29°C). Experiments continued until no experimental flies remained alive, at which time donor flies were discarded.

Starvation resistance assays. Following 48 h of exposure (see Exposure to donor flies), experimental flies were placed in fresh vials without donor flies containing 1% agar. For each assay, 50 experimental flies (in vials of 10 flies per vial) were established for all treatment/genotype groups. The number of dead flies in each vial was recorded every 2–4 h until no experimental flies remained alive.

Fat store (TAG) assays. Following 48 h of exposure, experimental flies were quickly frozen in a dry ice bath, then homogenized in 100 µl PBS/0.01% Triton-X in groups of 5 flies per sample, with 5–10 samples per treatment/genotype. Afterward, 5 µl of homogenate was added to 150 µl of Infinity Triglyceride Reagent (Thermo Electron) and incubated at 37°C for 10 min with constant agitation. Concentrations of TAG were determined by comparing the absorbance at 520 nm of experimental samples with known triglyceride standards.

Neurometabolomic analysis. Following 48 h of exposure, experimental flies were quickly frozen on ice, placed in a bath of ice and stored at ~80°C overnight. Heads were removed via vortex and separated from body parts by filtering through meshes. A total of 40–50 heads were then homogenized for 20 s in 200 µl PBS using the Fast Prep 24. Following the addition of 800 µl of methanol, samples were incubated for 30 min on dry ice, then homogenized again. The mixture was then spun at 13,000 rpm. for 5 min at 4°C, and the soluble extract was collected into vials. This extract was then dried using a speedvac at 30°C for approximately 3 h. Untargeted global aqueous metabolomics was performed in the Northwest Metabolomics Research Center at the University of Washington using an Agilent 6520 Q-TOF-MS coupled to an Agilent 1200SL ultra performance liquid chromatography system. This instrument has both electrospray and atmospheric pressure chemical ionization sources. Agilent Mass Hunter and Mass Profiler Professional software was used to identify and quantify metabolites and to analyse metabolite profiles.

Data imputation and PCA analysis. Using untargeted metabolomics, we were able to detect 1,463 metabolites for positive mode and 1,034 for negative mode in the first experiment, and 1,703 metabolites for positive mode and 1,365 for negative mode in the second experiment. After removing any metabolites missing from more than two samples (60–77%), we were left with 524 and 409 metabolites for positive and negative mode in the first experiment, and 392 and 379 metabolites for positive and negative mode in the second experiment, respectively. Metabolite abundance for remaining missing values in this data set were log-transformed and imputed using the k-nearest neighbour algorithm with the impute package of R Bioconductor (www.bioconductor.org). We then normalized the data to the standard normal distribution $\mu=0$, $\sigma^2=1$. PCA was performed using the mae package in Bioconductor. Metabolite abundances were visualized through the projection of samples on the principal components that significantly separate either mating or pheromone effects between different treatments. For each permutation, we randomly distributed the treatments to the real abundance of each metabolite. PCA was done for both randomized and real data. The degree of separation for each principal component was be measured by analysing between- and within-group variance based on the projection of samples on that principal component, which is indicated by the Z-score: $Z = \text{variance between groups}/\text{variance within groups}$, where variance between groups

$$Z = \sum_{k=1}^{N} \frac{n_k \times (\text{Mean}_{\text{post}} - \text{Mean}_{\text{pre}})^2}{\text{variance}}$$

where $N$ indicates the number of groups and $n_k$ indicates the number of samples in group $k$. The distribution of Z-score was obtained from 10,000 randomized data sets. Principal components that significantly deviated from this randomized distribution were considered a significant separation of groups. Individual metabolites whose abundances were associated with either mating or pheromone exposure were identified from positive and negative modes and combined for pathway enrichment analysis using MetaBolyst 3.0 (http://www.metaboanalysis.ca).

Temperature-dependent neuronal manipulations. For the corazonin manipulation experiments in which temperature was used to activate or inhibit crr-expressing neurons in adult flies, fly eggs from experimental and control flies were collected and reared in 18°C and 29°C for two generations. The Aedes aegypti mosquito was used to prepare 100% 29°C pupae. For these experiments, experimental flies were exposed to donor animals for 48 h, after which donor flies were removed and experimental flies were assayed. For lifespan experiments, experimental flies were exposed to donor animals beginning on day 2 following eclosion (after sexes had been separated), and exposure continued over the lifetime of the flies.

Complementary DNA was then synthesized using the Supernscript III first strand synthesis kit (Invitrogen). Quantitative real-time PCR was performed with SYBR green from SA Biosciences. Expression was normalized to expression of the housekeeping gene rp49. All reactions were performed in duplicate for technical replication. If no exponential amplification was observed, the sample was removed from analysis. The following primers were used:

- 4EBP forward: CGAACGGCACAAGCTGAGA
- 4EBP reverse: TTTCCGCTGGACGTGTAAGCA
- RP49 forward: ACCCTAAATGATACTGCGCAG
- RP49 reverse: CAAGGTGTCCCACTATCAGTCA

Circulating dILP2 enzyme-linked immunosorbent assays. Following exposure of flies carrying a FLAG-tagged dILP2 to the indicated donor flies for 48 h, haemolymph was extracted by skewering flies with a large needle, then immediately spinning them at 5,000 × g for 5 min at 4°C in a 0.6 ml tube perforated at the bottom with a 16 g needle, placed in a 2 ml tube. The haemolymph was collected from the bottom of the 2 ml encasing tube. Enzyme-linked immunosorbent assays were performed as described previously, using flies with FLAG-tagged dILP2.

Mating latency assays. To measure the influence of masculinization on female mating latency, we flipped individual Canton-S male flies into vials containing either a single control female ($w^{1118};UAS-TRA^{OK72+}$+), a single masculinized female ($w^{1118};OK72-Gal4/+;UAS-TRA^{OK72+}$+), or one control female and one masculinized female (that is, competition vials). The vial plug was located roughly 1 cm from the top of the food to limit space and promote interactions. Vials were monitored at least once every 10–15 min, and times to copulation and genotype of the mating female (for competition vials) were recorded when the male was seen to successfully mount the female. Likelihood-ratio tests were used to obtain $P$ values for the hypothesis of identical rates between treatments. Vials were observed for 6 h.

Fertilization assays. Fertilization assays were used to quantify successful mating rates of males with control and masculinized females. These experiments were performed by adding one male ($y^{w}$) and one female (either wild-type or masculinized) to a vial. Flies were then allowed to interact overnight (12 h), after which the male was removed. If evidence of larval development was not observed after 1 wk, it was assumed no successful fertilization occurred.
Offspring assays. To measure the lifetime offspring production, lifespan experiments were performed as described above, with the additional requirement that all vials within the assay period were saved and incubated at 25°C. Offspring were allowed to emerge for 2 wk, after which all flies were frozen and counted.

Video analysis. Eight-day-old, mated experimental yw male flies were lightly gassed and placed singly into 10 separate vials containing standard fly media and 5 donor flies (control male (w1118;OK2;UAS-TRA+;+), feminized male (w1118;OK2;Gu410/UAS-TRA+;+), control females (w1118;OK2;UAS-TRAEx11/+;+), or masculinized females (w1118;OK2;Gu410/UAS-TRAEx11/+;+)). After 24 h, flies in each vial were mouth pipetted into circular video arenas containing 5% sucrose and 2% agar. The arenas were placed into a 25°C incubator with 12 h light:12 h dark cycles and colour video cameras (placement of each treatment in the incubator was randomized). After 24 h, we collected 6 h of video from each treatment. The videos were analysed over a random 10 min interval where the total number of interactions (every time the experimental male touches, displays wing courtship song, and/or closely chases a donor fly) and the total interaction time (the total time the experimental male touches, displays wing courtship song and/or closely chases a donor fly) were quantified.

Wing damage assay. Five 8-day-old, mated experiment yw males with intact wings were lightly gassed and placed into 10 separate vials containing standard fly media and 25 donor flies (control male (w1118;OK2;UAS-TRA+;+), feminized male (w1118;OK2;Gu410/UAS-TRA+;+), masculinized male (w1118;OK2;Gu410/UAS-TRA+;+), or feminized male (w1118;OK2;UAS-TRAEx11/+;+). The vials were kept in a 25°C incubator with 12 h light:12 h dark cycles for 2 wk; fresh food was given every Monday, Wednesday and Friday during this exposure period. The flies were then anesthetized and the wing condition of each experimental male was assessed in a double blind fashion according to the following scale: 0 = males with no wing damage; 1 = males with >10% wing damage; 2 = males with 10–50% wing damage; 3 = males with >50% wing damage.

Statistics. Unless otherwise indicated, group- and pairwise-comparisons among survivorship curves (both lifespan and starvation) were performed using the D’Life computer software and the statistical software R. P values were obtained using Cox regression tests (select pairwise comparisons and group comparisons or interaction studies) as noted. For all box plots, box represents standard error of the mean (S.E.M., centred on the mean), whiskers represent 10%/90% and the mean of the mean (S.E.M., centred on the mean), whiskers represent 10%/90% and the

Data availability. Metabolomics data and analyses are provided as Supplementary File 1. All additional data are available upon request from the corresponding author.

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Author contributions

Z.M.H. and S.D.P. conceptualized the project, and Z.M.H., Y.L., D.E.L.P. and S.D.P. designed the experiments. Z.M.H., C.M.G. and J.C.J. performed the in vivo experiments, and Z.M.H., C.M.G. and S.D.P. analysed them. D.E.L.P. performed the metabolomics. Y.L. and S.D.P. analysed the metabolomics data, with input from Z.M.H. and D.E.L.P. S.K. created the NPF mutant used in Fig. 3. Z.M.H., Y.L. and S.D.P. wrote the manuscript, with comments from C.M.G. and D.E.L.P.

Additional information

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Competing interests

The authors declare no competing financial interests.