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Mifepristone/RU486 acts in *Drosophila melanogaster* females to counteract the life span-shortening and pro-inflammatory effects of male Sex Peptide

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Abstract

Males with null mutation of *Sex Peptide (SP)* gene were compared to wild-type males for the ability to cause physiological changes in females that could be reversed by mifepristone. Males from wild-type strains decreased median female life span by average -51%. Feeding mifepristone increased life span of these females by average +106%. In contrast, *SP*-null males did not decrease female life span, and mifepristone increased median life span of these females by average +14%, which was equivalent to the effect of mifepristone in virgin females (average +16%). Expression of innate immune response transgenic reporter (*Drosocin*-GFP) was increased in females mated to

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wild-type males, and this expression was reduced by mifepristone. In contrast, *SP*-null males did not increase *Drosocin*-GFP reporter expression in the female. Similarly, mating increased endogenous microbial load, and this effect was reduced or absent in females fed mifepristone and in females mated to *SP*-null males; no loss of intestinal barrier integrity was detected using dye-leakage assay. Reduction of microbial load by treating adult flies with doxycycline reduced the effects of both mating and mifepristone on life span. Finally, mifepristone blocked the negative effect on life span caused by transgenic expression of *SP* in virgin females. The data support the conclusion that the majority of the life span-shortening, immune-suppressive and pro-inflammatory effects of mating are due to male SP, and demonstrate that mifepristone acts in females to counteract these effects of male SP.

Keywords

life span; steroid; sexual conflict; sex peptide; innate immune response; mifepristone

Introduction

The drug mifepristone (RU486) is a synthetic steroid that antagonizes the activities of glucocorticoids and progesterone by binding to the type II glucocorticoid and progesterone receptors (Baulieu 1997; Chen et al. 2014). Mifepristone is used in humans as an abortifacient and as a treatment for Cushing's disease, and is being tested for its potential as an anti-cancer drug and for prophylactic birth control. Mifepristone has also been found to have pro-survival effects in immature mouse Purkinje neurons (Ghoumari et al. 2006; Rakotomamonjy et al. 2011).

We have recently reported that feeding mifepristone can dramatically increase the median life span of mated female *Drosophila melanogaster*, with lesser or no benefit for median life span in virgin females, and no benefit detected in males (Landis et al. 2015). In *Drosophila melanogaster* mating causes a variety of physiological changes in the female, including increased egg production, decreased receptivity to re-mating, altered food preference, decreased life span and increased expression of innate immune response genes, including antimicrobial peptide (AMP) genes (Feng et al. 2014; Ribeiro and Dickson 2010; Wolfner 2002). The increased reproduction and decreased life span are generally interpreted as an induced trade-off that may result from the diversion of metabolic resources towards reproduction and away from somatic maintenance pathways important for normal longevity. The changes caused by mating in the female are attributed to the action of male seminal proteins (Chapman et al. 1995), and several such proteins have been identified with both specific and overlapping effects (Findlay et al. 2014; Wolfner 2009). For example, the male seminal protein Ovulin stimulates the release of oocytes by the ovary (Heifetz et al. 2005). The majority of the effects of mating on female egg production and decreased receptivity to re-mating have been attributed to the male seminal protein called Sex Peptide (SP) (Chen et al. 1988; Feng et al. 2014; Liu and Kubli 2003; Tsuda and Aigaki 2016). SP produces the majority of its effects by binding to a specific G-protein coupled receptor called Sex Peptide Receptor (SPR) (Yapici et al. 2008), however some effects of SP may be independent of SPR (Hausmann et al. 2013). To determine if the effects of mifepristone in the mated

female are dependent upon the function of male SP, we assayed the ability of wild-type males and males that lack functional *SP* (called *SP*-null or *SPN* males) (Liu and Kubli 2003) to cause physiological changes in the female that could be reversed by mifepristone. In addition, the GAL4/UAS system (Brand and Perrimon 1993) was used to drive expression of *SP* in virgin females to allow assay of effects that could be reversed by mifepristone.

Materials and methods

Drosophila stocks, culture and life span assay

Drosophila were cultured on a standard agar/dextrose/corn meal/yeast media (Ren et al. 2009) at 25°C, and adult flies were passaged to fresh media every-other day. *Drosophila* strains are as previously described (Landis et al. 2015). The *Drosocin 826-A3 II* GFP reporter strain (abbreviated *Drosoc*-GFP) was generously provided by B. Lemaître and J.L. Imler (Tzou et al. 2000). Several strains were obtained from the Bloomington *Drosophila* Stock Center, including the *y[1] w[*]; P{w[+mC]=elav-Switch.O}GSG301* strain (abbreviated *y; Elav-GS*), strain *w[*]; P{w[+mC]=ppk-GAL4.G}2* (abbreviated *ppk-GAL4*), strain *w[1118]; PBac{w[+mC]=IT.GAL4}dsx[0598-G4]/TM6B, Tb[1]* (abbreviated *dsx-GAL4*), and the control strain *w[1118]*. The *w[1118]* strain is the isogenized version (*w[1118]-iso; 2-iso; 3-iso*) described by Ryder et al and sequenced by Platts et al (Platts et al. 2009; Ryder et al. 2004). All strains were cleared of any *Wolbachia* by three generations culture on doxycycline followed by >3 generations culture in absence of doxycycline prior to use (Ren et al. 2007). To generate female flies for life span experiments, males of the *w[1118]* strain were crossed to virgin females of the *y; Elav-GS* strain. The hybrid female progeny of these crosses were collected as virgins over 24 hours. These flies were either assayed as virgins, or were mated to young (1-2 weeks of age) males of the indicated genotype in vials at a ratio of 20 males to 20 females, for 48 hours, after which the males were removed. Unless indicated otherwise the genotype of the males used for mating was *w[1118]*. The *SP* null mutation strain *SP[0]/TM3,Sb* and *SP* deficiency strain *delta130/TM3,Sb* (Liu and Kubli 2003) were generously provided by M. Wolfner (Apgar-McGlaughon and Wolfner 2013). To generate *SP*-null males, males of the *SP* null mutation strain *SP[0]/TM3,Sb* were crossed to virgins of the *SP* deficiency strain *delta130/TM3,Sb* to generate trans-heterozygous *SP*-null male progeny, genotype *SP[0]/delta130*. The UAS-*SP* transgene strain (Nakayama et al. 1997) was generously provided by Todd Laverty and Barry Dickson. All ages are expressed as days from eclosion from the pupa at 25°C, and life span assays were conducted as previously described (Ford et al. 2007) with the following modifications. Flies were maintained as adults at ~20 flies per vial, with transfer to fresh media every other day. Mifepristone (RU486) (Sigma) was fed to adult flies at a final concentration of 160 µg/ml in the food media, and doxycycline ("DOX"; Sigma) at 640 µg/ml in the food media, prepared as previously described (Ren et al. 2009); for the experiments in Tables S10 and S11 mifepristone concentration was 200 µg/ml. Progesterone (Sigma), dopamine hydrochloride (Sigma) and L-DOPA (Sigma) were fed to adult flies at final concentration of 200 µg/ml. Median, percent change in median, log rank p values and ANOVA analyses were conducted using R statistical environment (R Development Core Team 2009).

Gompertz-Makeham equation modeling

Gompertz-Makeham (G-M) modeling was conducted using *WinModest* software (version 1.0.0.1) as previously described (Pletcher 1999; Shen et al. 2009). Briefly, for the G-M equation ($\mu_x = ae^{bx} + c$), the age-specific mortality rate (μ_x) was calculated using *WinModest* by binning the days over which deaths were counted (e.g., fly deaths were recorded every other day) such that $\mu_x = (-\ln(N_x + \delta_x N_x)) / \delta_x$, where N_x is the number of flies alive at day x , δ_x is the bin size, and μ_x is the mortality rate at day x . Parameters (a , b , c) were calculated by *WinModest* based on a likelihood ratio test. Microsoft Excel was used to plot the full model ($\mu_x = ae^{bx} + c$) as the trend line, along with data points indicating the calculated values for $\ln(\mu_x)$ at each age time point. Re-composed survival curves were generated by fitting the data to the Gompertz equation ($\mu_x = ae^{bx}$), i.e., omitting any age-independent mortality corresponding to G-M parameter c . The re-built survival curves were plotted using Microsoft Excel, using the reverse calculation of μ_x as follows: $\mu_x = ae^{bx}$, $P_x = e^{-\mu_x}$, where P_x was the probability of surviving from age (x) to age ($x+1$). Percent survival at each day (L_x) was calculated as $L_x = (L_{x-1})(P_x)$. For the re-composed survival curves, any value below 0.5% survival was considered to be the final data point.

Feeding assay

The capillary-feeding assay (CAFÉ) was conducted essentially as previously described (Ja et al. 2007). Briefly, age-synchronized female flies of the indicated hybrid genotypes were mated to *w[1118]* males as described above, and then maintained in the presence and absence of 160 $\mu\text{g/ml}$ mifepristone/RU486 in the media, with passage to fresh media every other day. At 12 days of age the flies were placed in culture vials, at 4 flies per vial, with a single Kimwipe placed at the bottom of the vial and thoroughly wetted with deionized water. Graded capillary tubes (three per vial) were inserted into the top of the vial through the stopper, and filled with liquid media, +/- supplementation with mifepristone/RU486 at 160 $\mu\text{g/ml}$. After acclimation to the vials for 24 hours, the total volume of media ingested by the flies was measured over 24 hours (11AM to 11AM). Seven replicate vials were assayed per group, and data are presented as average +/- standard deviation of volume ingested per fly per day. Liquid media was 5% sucrose, 5% yeast extract, 2.5% FD&C Blue No. 1 in deionized water.

Intestinal barrier integrity (“SMURF”) assay

Intestinal barrier integrity was assessed using the dye-leakage (“SMURF”) assay, essentially as previously described (Rera et al. 2011). Briefly, flies were maintained overnight (16 hours) on media that had been adjusted to 2.5% (weight/volume) FD&C Blue No. 1 dye. The flies were then scored under the dissecting microscope for tissue distribution of blue dye; presence of blue dye in tissues of thorax and head indicated loss of intestinal barrier integrity (“SMURF” fly; example shown in Fig S1).

Quantification of microbial load

The total aero-tolerant internal microbial load of flies was quantified essentially as previously described (Ren et al. 2007). Briefly, flies at 12 days of age were assayed using 4 flies per extract, and three extracts per group. Flies were surface sterilized by washing in

70% ethanol for 2 minutes, rinsed twice for two minutes using sterile PBS, and then homogenized in 300 μ l sterile PBS. Extracts were diluted 1:100 (or 1:1,000) with sterile PBS and 100 μ l of diluted extract was plated on nutrient agar plates. Plates were incubated at 25°C for 48-72 hours and total colony number was recorded. Data are presented as average $\pm$ standard deviation of bacterial count per fly, on log scale.

Microscopy and GFP quantification

Visible images, GFP fluorescence images, and image overlays for flies were generated using the Leica MZFLIII fluorescence stereomicroscope and *Spot* software as previously described (Tower et al. 2014). GFP fluorescence was quantified using captured GFP images and NIH *Image J* software, with mean and standard deviation calculated using 6 flies per sample (Tower et al. 2014). GFP was also quantified in groups of flies using GFP fluorescence video, essentially as previously described (Ardekani et al. 2012), by the USC Video Tracking Core facility (<http://dornsife.usc.edu/towerlab/uscvtc/>). Briefly, 4 replicate vials of 6 flies each were quantified for each group (virgin females, mated females, and mated females fed mifepristone). Four minute videos were recorded for each group of 6 flies at 60 frames per second using two synchronized cameras. *FluoreScore* software was used to track the flies in the videos, quantify their green pixel intensities, combine the data from the two cameras, and generate the sum of green pixel intensities for the group of flies for each frame; frames in which no flies were detected were discarded. The data were log transformed and the R statistical environment was used to calculate median and to conduct statistical analyses.

Results

Mifepristone acts in females to prevent the life span-shortening effect of male Sex Peptide

The genotype chosen for use as the virgin females is a hybrid genotype previously found to have both a long starting life span (median life span $\sim$ 70 days; see Table 1) and an especially robust response to mating (Landis et al. 2015)(progeny of *w[1118] X y; Elav-GS*). Males of three genotypes with a wild-type *SP* gene (*w[1118]*, *Oregon-R* and *Canton-S*) and males of the *SP*-null genotype were assayed for the ability to cause a reduction in the life span of females due to mating, in two independent experiments. In each experiment mifepristone was assayed for effects on life span for each group of females. A 3-way ANOVA analysis (Table S1) showed that males reduced life span of females ($F(4, 1912) = 109.81$, $p < 0.001$), that RU486 suppressed this effect ($F(1, 1912) = 374.56$, $p < 0.001$), and the RU486 by type of male interaction showed that this effect was not observed in all strains ($F(4, 1912) = 18.83$, $p < 0.001$).

The life span changes observed were of relatively large magnitude. Males from the *w[1118]* control strain reduced female life span by -49% in the first experiment (Fig. 1a, statistical summary in Table 1) and by -62% in the second experiment (Table 1). Note that the log rank test p values for the pair-wise comparisons are significant only for $p < 0.0056$, per a Bonferroni correction for 9 tests ($0.05/9$), and should only be used to precise the results of the ANOVA. Males from the wild-type strain *Oregon-R* reduced female life span by -42% and -34%, respectively, and males from the wild-type strain *Canton-S* reduced female life

span by -54% and -57%, respectively. Females mated to each of the types of males were assayed for life span in the presence and absence of mifepristone feeding (Fig. 1a, statistical summary in Table 1). For females mated to *w[1118]*/control strain males the mifepristone increased female median life span by +94% and +71%, respectively. For females mated to *Oregon-R* wild type strain males the mifepristone increased female median life span by +95% and +84%, respectively. Finally, for females mated to *Canton-S* wild type males the mifepristone increased the female median life span by +120% and +150%, respectively. Neither progesterone (200µg/ml) (Table S2), nor dopamine (200µg/ml) nor L-DOPA (200µg/ml) (Table S3) could increase life span of mated females, thereby supporting the specificity of the effect of mifepristone.

Previously a dye-uptake assay was used to quantify feeding in mated and mifepristone-treated mated females at day 12, and either no effect or slight increase in food ingestion was observed, arguing against a dietary restriction mechanism (Landis et al. 2015). Here the more sensitive capillary-feeding assay (CAFÉ) was employed (Ja et al. 2007). The same genotype females as used above were examined (progeny of *w[1118]X y; Elav-GS*), as well as females of another hybrid genotype that gives a robust response to mating and mifepristone (progeny of cross *w[1118]; p53B[6]X w[1118]; rTA(3)E2*) (Landis et al. 2015). The day 12 time point was again chosen because our previous studies indicate the critical period for mifepristone is within the first 20 days of adult life (Landis et al. 2015), and day 12 is when survival curves first begin to diverge for virgin, mated, and mated plus mifepristone flies (Fig. 1a). Using the CAFÉ assay food intake was found to be either unchanged or slightly increased in the mifepristone-treated females (Fig. 1b), supporting the conclusion that life span increase does not result from dietary restriction.

To generate *SP*-null males for use in experiments, males of the *SP*-null mutation strain *SP[0]/TM3,Sb* were crossed to virgins of the *SP* deficiency strain *delta130/TM3,Sb* to generate trans-heterozygous *SP*-null male progeny, genotype *SP[0]/del ta130*. The *delta130* chromosomal deficiency is reported to uncover the *SP* gene, and no expression of SP protein was detected in flies bearing this deficiency using Western blot (Liu and Kubli 2003). Trans-heterozygous *SP*-null males were used to avoid any background mutations on the *SP[0]* chromosome that might disrupt fertility if made homozygous (Liu and Kubli 2003). The trans-heterozygous *SP*-null males were confirmed to be fertile by crossing them individually to multiple *w[1118]* virgin females in test crosses, and 100% (46/46) produced abundant progeny. When crossed to hybrid virgins the *SP*-null males had no significant effect on the life span of the mated females (Fig. 1a; Table 1), consistent with the conclusion that SP is responsible for all or most of the life span shortening effect of mating. Feeding mifepristone to females mated to *SP*-null males increased median life span of these females by average +14%, equivalent to the effect of mifepristone in virgin females (average +16%; Table 1; average changes presented in Table S4). Taken together these results indicate that the majority of the life span-extending effects of mifepristone in the mated females is dependent upon the life span-shortening effects of male SP.

Mifepristone acts in females to reduce the activation of a *Drosocin*-GFP reporter by male Sex Peptide

Both mating and male SP have previously been reported to cause up-regulated expression of innate immune response genes in the female, including antimicrobial peptide genes (AMP genes) (Peng et al. 2005). We have previously used high-throughput RNA sequencing analysis to identify increases in gene expression caused by mating that are in turn reversed by feeding mifepristone to the mated females, and among these genes were several AMP genes, including *Drosocin* (Landis et al. 2015). To ask if mifepristone could prevent the activation of an AMP gene by mating and male SP, the expression of a transgenic *Drosocin*-GFP reporter (Tzou et al. 2000) was assayed in female flies at multiple time points post-mating using GFP video quantification (Fig. 2a; Table S5) as well as GFP image capture and *Image J* quantification (Fig. 2b, c; Table S6). The expression of the *Drosocin*-GFP reporter was up-regulated by mating, and this up-regulation was reduced by feeding the female flies mifepristone. Moreover, up-regulation of *Drosocin*-GFP reporter was not observed in females mated to *SP*-null males (Fig. 2b, c; Table S6). The data indicate that SP is necessary for the up-regulation of the *Drosocin*-GFP reporter in mated females, and that this up-regulation is reduced or prevented by feeding mifepristone.

Mated females exhibit an increase in endogenous microbial load

The total internal aero-tolerant microbial load of flies was assayed by homogenizing surface-sterilized flies, and plating the extracts on nutrient agar plates, as previously described (Ren et al. 2007). Mated females showed increased internal microbial load relative to virgin flies (Fig. 2d), suggesting a loss of normal immune function and consistent with the inflammatory response shown above. In contrast, no significant increase in microbial load was detected in mated females fed mifepristone, or in females mated to *SP*-null males (Fig. 2d), indicating that mifepristone prevents the immune-suppressive and pro-inflammatory effects of male Sex Peptide. Parallel samples of flies were assayed for intestinal barrier integrity by feeding on media supplemented with blue dye. Leakage of blue dye from the gut into peripheral tissues indicates loss of normal intestinal barrier integrity (“SMURF” flies) (Rera et al. 2011); see example in Fig. S1). No loss of intestinal barrier integrity was detected in the mated females, with or without mifepristone feeding (Fig. S1), indicating that increased microbial load and increased immune reporter expression occur in the absence of any detectable loss of intestinal barrier integrity, at least as indicated by the dye-leakage (“SMURF”) assay.

Doxycycline (DOX) reduces microbial load and reduces the effects of mating and mifepristone

Treatment of adult flies with high concentrations of DOX in the media reduced endogenous microbial load by a factor of 10^3 (Fig. S2), consistent with our previous studies of antibiotics (Ren et al. 2007). DOX was assayed for its potential ability to alter the life span effects of mating and of mifepristone, in two independent experiments (Fig. 3a, b; Tables S7, S8). In the first experiment a three-way ANOVA indicated a significant effect of DOX treatment, and a significant interaction between mifepristone and DOX, and between genotype (virgin or mated) and DOX (Table S8). In the second experiment the three-way ANOVA indicated a

significant interaction between DOX and genotype, however no significant effect of DOX (Table S8); we note that three-way ANOVA confirms the effects of DOX when using transgenic expression of SP, as shown below (see Table S11). In the presence of DOX the effect of mating in the two experiments was reduced from -56% and -45% to -18% and -21%, respectively (Table S7). Similarly, the effect of mifepristone was reduced from +53% and +65% to +0% and +19%, respectively (Table S7). Taken together these data suggest that mating may reveal a pathogenic (life span shortening) effect of the endogenous bacteria. In mammals and humans the presence of pathogens is often associated with an increase in the initial mortality rate (Gompertz mortality rate parameter a) (Finch 1990). When *Drosophila* female life span data were previously fitted to the Gompertz-Makeham equation, mating was found to cause increased initial mortality rate (parameter a) and decreased mortality rate acceleration with age (parameter b) (Shen et al. 2017). Mifepristone feeding reversed those effects by causing decreased initial mortality rate and increased mortality rate acceleration with age. The inverse correlation between parameters a and b is known as the Strehler-Mildvan relationship (Strehler and Mildvan 1960). Here fitting data to the Gompertz-Makeham equation revealed that mating to *SP*-null males (Fig. 3C, Table S9) or treatment with high-concentration DOX (Fig. 3D, Table S9) also caused decreased initial mortality rate and increased mortality rate acceleration with age, thereby reversing the effects observed upon mating to wild-type males, and again indicating a Strehler-Mildvan relationship.

Mifepristone blocks the negative effect on life span caused by transgenic expression of SP in virgins

To confirm that the effects of mating and of mifepristone were due specifically to male SP, the GAL4/UAS system was used to drive expression of SP in virgin female flies. The UAS-*SP* strain was crossed to *w¹¹¹⁸* strain to generate control flies containing only the UAS-*SP* transgene (Fig. 4a). The UAS-*SP* strain was also crossed to *dsx-GAL4* driver (Fig. 4b) and *ppk-GAL4* driver (Fig. 4c) to drive expression of UAS-*SP* in *dsx*-positive and *ppk*-positive cells, respectively. These drivers were chosen because *dsx*-positive and *ppk*-positive neurons in the genital tract express SPR, and transgenic expression of SP in these cells has previously been shown to recapitulate the increased oviposition (egg laying) and reduced receptivity to re-mating normally caused by mating and SP (Hausmann et al. 2013; Rezaval et al. 2014; Rezaval et al. 2012; Yang et al. 2009). For each genotype life span was assayed in virgin flies in the presence (red diamonds) and absence (blue circles) of mifepristone, in two independent experiments (Fig. 4 and Tables S10, S11). Mifepristone had no effect on life span in the control flies containing only the UAS-*SP* transgene (Fig. 4a). Driving expression of UAS-*SP* with either *dsx-GAL4* (Fig. 4b) or *ppk-GAL4* (Fig. 4c) caused decreased life span relative to controls, and this negative effect was rescued by feeding the flies mifepristone, thereby yielding large magnitude life span increases due to mifepristone. A two-way ANOVA indicates the significant effect of mifepristone, the significant difference between genotypes, and a significant interaction between mifepristone and genotype (Table S11). These results support the conclusion that mifepristone acts in females to prevent the negative effect on life span caused by SP. The experiment was repeated, with and without feeding the adult flies DOX to reduce microbial load (Tables S10, S11). A three-way ANOVA for the second experiment indicates a significant effect of mifepristone, a

significant effect of DOX, and a significant difference between genotypes (Table S11). In addition there was a significant interaction between mifepristone and genotype, between mifepristone and DOX, and between DOX and genotype (Table S11). Taken together, these results show that in the presence of high concentration DOX both the negative effect of UAS-*SP* and the positive effect of mifepristone were reduced or eliminated, consistent with the results obtained above with mated flies, and indicating a requirement for bacteria to observe the negative effects of *SP* and the positive effects of mifepristone.

Discussion

Mating causes a variety of physiological changes in the female, including increased oogenesis, decreased life span, increased expression of innate immune response genes and decreased receptivity to re-mating (Wolfner 2002). Several of these physiological changes in the female have previously been demonstrated to be caused entirely or in part by male *SP*, including increased oogenesis, decreased receptivity to re-mating, and increased immune gene expression (Aigaki et al. 1991; Chapman et al. 2003; Chen et al. 1988; Liu and Kubli 2003; Peng et al. 2005).

We have previously reported that mifepristone (RU486) acts in female flies to decrease the negative effect on life span caused by mating, and also reduces progeny production by the mated females (Landis et al. 2015). Here experiments were conducted to determine if mifepristone acts in female flies to counteract the effects of male Sex Peptide (*SP*). Wild-type males were compared to *SP*-null mutant males for the ability to cause physiological changes in the female that could be reversed by mifepristone. The majority of the life span effects of mifepristone in the female were found to be dependent upon wild-type *SP* gene function in the male, indicating that mifepristone acts in females to counteract the life span shortening effects of male *SP*. Feeding mifepristone increased life span of females mated to wild-type males by average +106%. In contrast, *SP*-null males did not decrease female life span, and mifepristone increased median life span of these females by average +14%, which was equivalent to the effect of mifepristone in virgin females (average +16%). Our studies indicate that the positive effect of mifepristone on virgin life span is observed only when using the longest-lived starting strains (Landis et al. 2015; Shen et al. 2017); we speculate that *SP* may activate a trade-off between reproductive metabolism versus longevity that is already partially active in the virgin female. Previously the effect of mating and mifepristone was examined using several wild-type and transgenic female genotypes, both as homozygotes and in hybrid crosses, and greater effects were observed in the genotypes with the longest starting life spans (Landis et al. 2015; Shen et al. 2017). The reasons for this variability in response to mating and mifepristone is not yet clear, but may be related to variability in the associated microbiome, as discussed further below.

Recently mifepristone has been reported to have an aversive effect on feeding when using low-nutrient media, but to have no significant effect when using normal-nutrient media (Yamada et al. 2017). Consistent with that result, the CAFÉ feeding assay employed here showed no decrease in food intake in the presence of mifepristone (Fig. 1b). Indeed, in both our previous dye-feeding assays (Landis et al. 2015) and the present CAFÉ assays, food intake was sometimes significantly greater in the presence of mifepristone; we speculate this

may be due to the generally greater health of the mifepristone-fed flies. Taken together, the feeding assays indicate that the mifepristone life span increase is not due to dietary restriction caused by reduced food intake. However, an effect of mifepristone on specific nutrient absorption and/or nutrient processing by the microbiota remains among the several interesting possible mechanisms for mifepristone action in the mated female fly.

The innate immune-response transgenic reporter *Drosocin*-GFP was up-regulated by mating, and this was dependent upon male *SP* and reversed by feeding females mifepristone. In addition, the endogenous microbial load was increased by mating, and this effect was dependent upon male *SP* and reversed by feeding mifepristone. These results indicate that mifepristone acts in females to counteract the pro-inflammatory and immune-suppressive effects of male *SP*. Previously male *SP* has been shown to reduce the ability of the female to resist a pathogenic bacteria (*Providencia rettgeri*) introduced into the circulatory system by piercing the cuticle with a needle (Short et al. 2012). The data presented here show that male *SP* also causes a loss of female control over the normal endogenous bacteria, and that this effect is reduced by mifepristone. Feeding the antibiotic DOX to adult flies greatly reduced endogenous bacterial load, and this reduced the effects of mating, mifepristone and *SP* on life span. Taken together the results indicate a role for bacteria in the life span effects of mating, male *SP* and mifepristone.

In mammals and humans environmental pathogens typically decrease life span by increasing the initial mortality rate (Gompertz parameter *a*) (Finch 1990). Here data were fitted to the Gompertz-Makeham equation to analyze mortality rates for several conditions. Male *SP*-null mutation and DOX both increased median life span of mated females by decreasing initial mortality rate (Gompertz parameter *a*), consistent with a role for a bacterial pathogen. The *WinModest* software uses a maximum likelihood model to estimate Gompertz parameters, and yielded statistically significant differences for the parameters in these experiments (Table S9); however, we note that the results are constrained by the relatively small sample sizes employed (85-95 flies for each sample) (Promislow et al. 1999). The decrease in parameter *a* correlated with an increase in the mortality rate acceleration with age (Gompertz parameter *b*), such that the increase in median life span observed was not associated with a significant increase in maximum life span. The inverse relationship between Gompertz parameters is often referred to as the Strehler-Mildvan relationship, based on its first report (Strehler and Mildvan 1960). Previously both mating and mifepristone life span effects were found to exhibit a robust Strehler-Mildvan relationship across multiple genotypes (Shen et al. 2017). It has been reported that the Strehler-Mildvan relationship can sometimes result from an artifact of the model fitting process, when the Gompertz equation is used instead of the Gompertz-Makeham equation (Gavrilov and Gavrilova 1991), and when the magnitude of life span changes is small (Tarkhov et al. 2017). However, in our previously published (Shen et al. 2017) and present studies the Gompertz-Makeham equation was employed, and life span changes were large in magnitude, indicating that a Strehler-Mildvan relationship is indeed associated with the life span effects of mating, mifepristone and DOX. It has been suggested that the inverse relationship between parameter *a* and parameter *b* revealed by Gompertz-Makeham modeling be referred to as the compensation law of mortality (Gavrilov and Gavrilova 1991). One possibility suggested by the present data is that the Strehler-Mildvan relationship (or compensation) is most robustly observed when life span is being

altered by bacterial pathogens. In the future it will be of interest to further explore the Strehler-Mildvan relationship and its possible biological implications.

To confirm that the effects of mifepristone are dependent upon male *SP*, the *SP* transgene was expressed in virgin females to determine effects that could be reduced by mifepristone. Transgenic expression of *SP* in virgin females decreased life span and this was reversed by feeding mifepristone. The life span effects of transgenic *SP* and of mifepristone were reduced by DOX, again consistent with a role for bacteria. Taken together the data indicate the mifepristone acts in females to reduce or eliminate several effects of male *SP*, including effects on life span, inflammation and immune function.

The negative correlation between life span and endogenous bacterial load observed here suggests that male *SP* increases reproduction at the cost of immune function, and that life span is decreased only when sufficient bacteria and/or the appropriate bacterial species are present to become pathogenic. This may be one explanation for why the life span effects of mating and of mifepristone were observed to vary across different *Drosophila* genotypes (Landis et al. 2015), as only some strains may be associated with the requisite bacterial species. The endogenous *Drosophila* microbiota (Broderick and Lemaitre 2012) has been suggested to become pathogenic in females under other conditions, including advanced age, non-optimal diet or mutation of immune response genes (Clark et al. 2015; Erkosar and Leulier 2014; Rera et al. 2012). Certain studies also observed a correlation between bacterial load and loss of intestinal barrier integrity as revealed by the dye-feeding (“SMURF”) assay (Clark et al. 2015; Rera et al. 2012). Interestingly, a loss of intestinal barrier integrity was not observed here, indicating that the increased microbial load, inflammation and increased mortality due to mating and male *SP* occur in the absence of any detectable loss of intestinal barrier integrity, at least as indicated by the dye-feeding (“SMURF”) assay. The potential mechanism(s) for bacterial toxicity in the presence of an intact intestinal barrier function include bacterial metabolites, and this will be an interesting area for future research.

The large magnitude effects on median life span observed here for male *SP* and for mifepristone benefit from our identification of hybrid genotypes that exhibit a particularly robust response to mating (Landis et al. 2015). In contrast to the results presented here, certain previous studies found limited evidence for a requirement for *SP* gene function in the male to cause decreased median life span in mated females. One previous study examined males where *SP* expression had been reduced in the male using RNAi (called *SP* knock-down males) (Wigby and Chapman 2005). That study found that *Dahomey* strain females mated to *SP* knock-down males for 48 hours had median life span equivalent to females mated to wild-type males, indicating that *SP* did not have a significant effect on female life span under those conditions. One possible explanation might be that in those experiments the effects of mating were masked by the short starting life span of the females (median approximately 30 days), as our previous studies indicate that the negative effects of mating are most apparent in long-lived starting strains (Landis et al. 2015). In another previous study, the *SP* null mutation strain *SP[0]* and *SP* deficiency strain *delta130* were each backcrossed to the *Dahomey* wild-type background, and the resulting strains were then crossed to each other to generate *SP*-null males. Those *SP*-null males were found to decrease the median life span of *Dahomey* strain females to the same extent as did wild-type males,

indicating that SP was not required for mating to decrease median female life span under those conditions (Fricke et al. 2014). The reasons for the difference between those results and our present results are not yet clear, but possibilities include the possible loss of a *SP* mutation during backcrossing, or differences in the way different female genotypes respond to male SP, potentially as affected by the microbiota.

Because mifepristone is a steroid hormone antagonist, the ability of mifepristone to counteract the effects of SP suggests that steroid hormone signaling may be a downstream effector of the actions of SP (model in Fig. 5). One logical candidate for the relevant steroid hormone is ecdysone. Ecdysone has been shown to regulate *Drosophila* oogenesis and a female metabolic state that favors reproduction (Ameku and Niwa 2016; Belles and Piulachs 2015; Sieber and Spradling 2015), as well as to modulate adult life span (Simon et al. 2003; Tricoire et al. 2009). Indeed, SP is reported to induce octopamine signaling in the mated female (Rezaval et al. 2014), and monoamines including octopamine and its precursor tyramine are implicated in the regulation of steroid hormone production (Ohhara et al. 2015). In the future it will be of interest to determine if mifepristone counteracts the effects of mating by acting as an antagonist of ecdysone, and/or whether it acts by counteracting other signaling mechanisms downstream of SP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SP	Sex Peptide
SPR	Sex Peptide Receptor
SPN	Sex Peptide null
AMP	Anti-microbial peptide

GFP Green fluorescent protein

DOX Doxycycline

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Fig. 1.

Life span effects of mifepristone in the mated female are dependent upon *Sex Peptide* gene function in the male. **a** Survival curves. Virgin females and females mated to males of the indicated genotypes were maintained as adults in the presence (+) and absence (-) of mifepristone. Statistical summary for this experiment and an independent replicate experiment are presented in Table 1 and Table S1. Or-R is *Oregon-R* males. SPN is *SP*-null males. **b** CAFÉ feeding assay. Mated females of the indicated hybrid genotypes were cultured in presence (+) and absence (-) of mifepristone/RU486 and assayed for volume of intake of liquid food (-/+ mifepristone) at 12 days of age. Data are plotted as average $\pm$ standard deviation of volume ingested per fly. Statistically significant difference of (+) relative to (-) for each genotype was determined using unpaired, two-sided t-tests and difference of $p < 0.05$ is indicated with asterisk

Fig. 2.

Mifepristone acts in females to counteract the pro-inflammatory and immune-suppressive effects of male SP. **a-c** Transgenic immune reporter expression. **a** *Drosocin*-GFP reporter flies were assayed for GFP fluorescence at day 7 (upper panel) and at day 30 (lower panel) using GFP-video quantification. V, virgin. M, mated to *w[1118]* males. M+RU486, mated to *w[1118]* males and maintained in presence of mifepristone/RU486. Data are presented as box-plots, with horizontal line indicating median. Y-axis is mean GFP intensity per frame in relative units. M and M+RU486 groups were compared to V group at each time point using unpaired, two-sided t-tests, and $p < 10^{-6}$ for each comparison (Table S5). Bonferroni correction was applied to determine significance ($0.05/4 \text{ tests} = 0.0125$), and significant differences are indicated by asterisk. **b, c** *Drosocin*-GFP reporter flies were assayed at day 30 for GFP expression using fluorescence microscopy, and fluorescence was quantified using image capture and *Image J* software. Flies were virgin, mated, mated plus mifepristone/RU486, and mated to *SP*-null males (SPN), as indicated. **b** Immune reporter image capture. Overlay of visible light image and GFP fluorescence image for a representative fly from each group. For the mated group the brightest fly is presented to show details of tissue distribution of fluorescence. **c** Quantification of immune reporter image capture. GFP fluorescence of *Drosocin*-GFP reporter flies was quantified from captured images using *Image J*, $N=6$ flies for each group, plotted as average $\pm$ standard deviation. Y-axis is mean GFP intensity per fly in relative units. Oneway ANOVA analysis is

presented in Table S6. Asterisks indicate statistically significant difference compared to virgin sample ($p < 0.016$) using unpaired, two-sided t-test. Bonferroni correction was applied to determine significance ($0.05/3 \text{ tests} = 0.016$). **d** Endogenous microbial load. The total internal aero-tolerant microbial load was quantified by plating fly extracts at 12 days of age. The average $\pm$ standard deviation is plotted on log scale. Asterisks indicate statistically significant difference compared to virgin sample ($p < 0.016$) using unpaired, two-sided t-test. Bonferroni correction was applied to determine significance ($0.05/3 \text{ tests} = 0.016$), and significant differences are indicated by asterisk. V, virgin. M, mated to *w¹¹¹⁸* males. SPN, mated to *SP*-null males. (-) minus mifepristone feeding. (+) plus mifepristone feeding

Fig. 3.

Reducing bacteria with DOX reduces the effect of mating and of mifepristone on life span.

a, b Effect of high concentration DOX on adult life span. Control cross females were assayed as virgins (V), or mated to *w[1118]* males (M), in presence (+) and absence (-) of mifepristone/RU486 in the media, as indicated. Statistical summary for this experiment and an independent replicate experiment in Tables S7, S8. **a** Without DOX treatment. **b** With DOX treatment. **c, d** Gompertz-Makeham modelling of survival data. Left panels are the plot of natural log mortality rate versus time, and the trend line for data fitted to the Gompertz-Makeham model. Right panels are reconstructed survival curves for data fitted to the Gompertz-Makeham model, omitting the age-independent mortality parameter *c*. **c** Comparison of females mated to *w[1118]* males (data from Fig. 1, *n* = 95) to females mated to *SP*-null males (data from Fig. 1, *n* = 96). Mating to *SP*-null males caused decreased parameter *a* and increased parameter *b* relative to mating to *w[1118]* males. **d** Effect of DOX. Maintaining mated females in presence of DOX (data from panel **b**; *n* = 93) causes decreased parameter *a* and increased parameter *b* relative to mated females maintained in absence of DOX (data from panel **a**; *n* = 84). Statistical summary for Gompertz-Makeham modeling is presented in Table S9.

Fig. 4.

Mifepristone blocks the negative effect on life span caused by transgenic expression of SP in virgin females. Survival curves are presented for virgin females maintained in absence of mifepristone/RU486 (- ; blue circles) and in presence of mifepristone/RU486 (+ ; red diamonds). **a** Control flies, progeny of cross *w[1118] X UAS-SP*. **b** Experimental group flies, progeny of cross *dsx-GAL4 X UAS-SP*. **c** Experimental group flies, progeny of cross *ppk-GAL4 X UAS-SP*. Statistical summary for this experiment and independent replicate experiment is presented in Tables S10, S11.

Fig. 5.

Summary model. In *Drosophila* females, mating and male Sex Peptide cause physiological changes including increased progeny production, inflammation, reduced immune function and reduced life span. These changes can be blocked by feeding the females the steroid hormone antagonist mifepristone/RU486, thereby implicating steroid hormone signaling. The tradeoff is associated with specific changes in gene expression, as previously revealed by high-throughput RNA sequencing, including down-regulation of the dosage compensation regulator *Unr* and up-regulation of innate immune response genes, including AMP genes. The data suggest a trade-off wherein resources are diverted to female-specific metabolism and reproduction at the expense of immune function and longevity

Table 1

Statistical summary for life span effects of mating to wild-type males and to *SP*-null males. Life span curves for Experiment 1 are presented in Fig 1A. The (-) drug flies for each cross are compared to (-) drug virgins to show effect of mating; the (+) drug flies for each cross are compared to (-) drug flies for that cross to show effect of mifepristone. Three-way ANOVA presented in Table S1. Life span units are days post-eclosion from the pupa.

Genotype	RU486	Sample Size	Mean (SD)	Median	90% Mortality	Change in Mean (%)	Change in Median (%)	Log-Rank Test (p)
Experiment 1								
virgins	-	96	64 (23)	66	94			
virgins	+	95	76 (27)	84	103	+19.4	+27.3	1.24E-6
X <i>w/1118</i> /males	-	95	40 (21)	34	69	-38.1	-48.8	4.03E-12
X <i>w/1118</i> /males	+	97	65 (26)	66	100	+65.1	+94.1	7.44E-13
X <i>Or-R</i> males	-	91	44 (26)	38	88	-30.9	-42.4	0.0017
X <i>Or-R</i> males	+	97	77 (24)	74	106	+73.2	+94.7	6.20E-11
X <i>Canton-S</i> males	-	94	39 (24)	30	73	-38.8	-54.5	9.23E-7
X <i>Canton-S</i> males	+	98	64 (26)	66	100	+64.3	+120	1.33E-7
X <i>SP</i> -null males	-	96	67 (20)	70	94	+5.15	+6.06	0.5549
X <i>SP</i> -null males	+	98	85 (20)	87	105	+25.8	+24.3	4.38E-9
Experiment 2								
virgins	-	99	70 (21)	74	94			
virgins	+	94	78 (16)	76	100	+11.0	+2.70	0.0438
X <i>w/1118</i> /males	-	101	34 (19)	28	60	-51.8	-62.2	0.0000
X <i>w/1118</i> /males	+	101	50 (22)	48	78	+47.6	+71.4	6.07E-7
X <i>Or-R</i> males	-	90	48 (24)	49	82	-31.2	-33.8	1.69E-7
X <i>Or-R</i> males	+	93	83 (23)	90	100	+72.9	+83.7	0.0000
X <i>Canton-S</i> males	-	102	41 (25)	32	80	-41.3	-56.8	9.42E-10
X <i>Canton-S</i> males	+	97	70 (32)	80	104	+69.9	+150	3.30E-12
X <i>SP</i> -null males	-	99	75 (21)	80	98	+7.66	+8.11	0.0724
X <i>SP</i> -null males	+	99	79 (20)	82	96	+4.86	+2.50	0.4631