Transgeneratonal inheritance of ethanol preference is caused by maternal NPF repression.

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Summary

Rapid or even anticipatory adaptation to environmental conditions can provide a decisive fitness advantage to an organism. The memory of recurring conditions could also benefit future generations, however neuronally-encoded behavior isn't thought to be inherited across generations. We tested the possibility that environmentally triggered modifications could allow "memory" of parental experiences to be inherited. In *Drosophila melanogaster*, exposure to predatory wasps leads to inheritance of a predisposition for ethanol-rich food for five generations. Inhibition of Neuropeptide-F (NPF) activates germline caspases required for transgenerational ethanol preference. Further, inheritance of low NPF expression in specific regions of F_1 brains is required for the transmission of this food preference: A maternally derived *NPF* locus is necessary for this phenomenon, implicating a maternal epigenetic mechanism of NPF-repression. Given the conserved signaling functions of NPF and its mammalian NPY homolog in drug and alcohol disorders, these observations raise the intriguing possibility of NPY-related transgenerational effects in humans.

Introduction

To what extent is personality and behavior predetermined at birth? Philosophers and scientists alike have struggled with this question, and many have settled on the *tabula rasa*, or *blank slate* perspective. This long-standing notion posits we are without form or direction until our individual experiences shape us. Over the past decades however, evidence has accumulated that suggests parental environment can have significant phenotypic consequences on the next generation, thus eroding this notion of a blank slate. The Dutch Hunger Winter Study was one of the first documented examples of ancestral experiences influencing subsequent generations. Children conceived in the Netherlands during the World War II blockade, and ensuing famine, had higher rates of obesity and diabetes (Heijmans et al., 2008; Schulz, 2010; Stein, Susser, Saenger, & Marolla, 1975). More recent studies have found that neurological and mental health conditions also appear to have persistent impact on the next generations (Yeshurun & Hannan, 2018). Further, risk factors for children of Holocaust survivors, such as reduced cortisol sensitivity has been linked to methylation state of the glucocorticoid receptor promoter, and increased methylation in offspring was associated with paternal diagnosis of posttraumatic stress disorder(Yehuda et al., 2014).

Studied largely in the public health context, there are limited examples of environmental inheritance that can be experimentally tested. Genetic model systems thus are indispensable for understanding molecular mechanisms of causation. For example, male mice trained to associate fear with an odor-transmitted sensitivity of this odor to their sons. In this instance, researchers concluded that offspring possessed an increased abundance of sensory neurons specific to the same odor their fathers were trained to fear

(Dias & Ressler, 2014). Similarly, environmental enrichment activities can ameliorate behavioral defects of mutant mice defective in long-term potentiation and memory, and this behavioral rescue is heritable to the next generation through the activation of an otherwise latent p38 signaling cascade (Arai, Li, Hartley, & Feig, 2009). Parental exposure to toxins and nutritional challenges also can change germline information, affecting growth and metabolism of future generations(Carone et al., 2010; Chen et al., 2016; Sharma et al., 2016; Skinner et al., 2013). These few examples suggest that parental environment can have a profound impact on subsequent generations. Elucidating mechanisms behind these environmentally triggered epigenetic programs is essential for a complete understanding of the foundational principles upon which biological inheritance is based.

Drosophila melanogaster females, when cohabitated with endoparasitoid wasps, shift to prefer ethanol food as an egglaying substrate, where ethanol food protects Drosophila larvae from wasp (Kacsoh, Lynch, Mortimer, & Schlenke, 2013a). *Drosophila suzukii* similarly shifts egglaying preference to food with atropine, giving its progeny protection against wasp(Poyet et al., 2017). Ethanol preference in *D. melanogaster* is linked to a decrease in Neuropeptide F (NPF) in the female brain (Kacsoh et al., 2013a), consistent with previous work on NPF (Shohat-Ophir, Kaun, Azanchi, Mohammed, & Heberlein, 2012), and its mammalian homolog NPY studied in the context of drug addiction (Gonçalves, Martins, Baptista, Ambrósio, & Silva, 2016; Landayan & Wolf, 2015). NPY modulation governs ethanol consumption in rats (Thiele, Marsh, Marie, Bernstein, & Palmiter, 1998) and is implicated in human alcohol abuse disorders(Mayfield et al., 2002; Mottagui‐Tabar et al., 2005). This behavioral

output is believed to be a consequence of the NPF/NPY role in the rewards pathway, with NPF signaling being inherently rewarding (Desai, Upadhya, Subhedar, & Kokare, 2013; Shao et al., 2017). NPF activity is considered representative of the motivational state of the fly (Krashes et al., 2009; Landayan & Wolf, 2015). Several recent studies also have shown that 'stressful' experiences regulate NPY/NPF levels, providing a link between environmental cues and NPF/NPY signaling (Broqua, Wettstein, Rocher, Gauthier-Martin, & Junien, 1995; Sah et al., 2009; Shohat-Ophir et al., 2012). Here we present findings that link maternal environmental conditions to cause inheritance of an altered reward pathway *via* depressed NPF signaling and preference for ethanol.

Results

Inheritance of ethanol preference

Drosophila were cohabitated with female wasps for four days, then separated and flies were placed into embryo collection chambers for 24 hours. Embryos were divided into two cohorts and each developed in the absence of adult flies or wasp. One cohort was used to propagate the next generation and never treated to ethanol food; the second cohort was used in the ethanol preference assay and then discarded (Fig. 1a).

Wasp-exposed Canton-S flies lay approximately 94% of their eggs on ethanol food (Fig. 1b). This behavior persists in their offspring despite the F_1 generation never having direct interaction with wasps (Fig. 1b). Ethanol preference in F_1 was less potent, with 73% of the eggs laid on ethanol food ($p = 8.6e^{-7}$, Table S1). Remarkably, this inherited ethanol preference persisted for five generations, gradually reverting back to the mock exposed baseline (Fig. 1b). These observations were replicated in an additional

93 wild type Oregon R (OR) strain (Table S2), suggesting that the phenomenon is not
94 specific to a particular genetic aberration or background. This indicates that inheritance
95 of ethanol preference is not a permanent germline change, but rather it is a reversible
96 trait. Ethanol preference was measured for two days for initial experiments (Fig. 1-3 &
97 S1); day one and day two showed similar trends, suggesting that flies do not habituate to
98 ethanol nor does the preference fade over the course of the experiment (Table S3).
99 Confirming previous findings, following a wasp exposure F_0 flies have an ethanol
100 preference that persists for more than a week, returning to baseline after ten days (Fig.
101 S1a). Sister cohorts of F_1 flies were collected at two time points along this F_0 ethanol
102 preference decay; one immediately following wasp exposure (brood 1), and the second
103 ten days post wasp exposure (brood 2). Brood 2 did not display an inherited ethanol
104 preference, suggesting that wasp exposure does not inflict a permanent change in the F_0
105 germline (Fig. S1d). Again, these findings replicated in OR flies (Table S2), indicating
106 that these observations are robust and not dependent on the context of a particular genetic
107 background.
108 To explore further the role of time and dynamics of wasp exposure, multiple
109 generations of flies were exposed to wasps. We found that inherited ethanol preference
110 can be enhanced with successive generations of wasp exposure (Fig. S1e). This trend did
111 not repeat when nonconsecutive generations were repeatedly exposed to wasps (Fig. S1f).
112 This suggests that the enhancing effect observed in the successive exposures is time
113 sensitive and may be linked to the ethanol preference of the parental flies.
114 To explore the required neural signaling to the germline, mutants defective for
115 long-term memory were assayed. Previous studies have shown that flies defective in

116 long-term memory exhibit an ethanol preference only in the presence of wasps but not
117 after wasp removal. The long-term memory mutant *Orb2*$^{\Delta Q}$ produced offspring with an
118 ethanol preference when the embryo collection was conducted in the presence of wasps,
119 but this ethanol preference was greatly reduced in offspring collected post-wasp exposure
120 (Fig. 1c). These data provide insight in two ways. First, functional long-term memory is
121 not a compulsory requirement to generate ethanol-preferring offspring. Secondly, intact
122 long-term memory is not required to inherit ethanol preference. Given that ethanol
123 preference in the absence of wasps is long-term memory dependent, this experiment
124 reveals that the neuronal signaling is different for maintained ethanol preference in the F_0
125 and F_1 flies(Bozler et al., 2017).

126 Several other factors point to distinctions between the F_0 and F_1 ethanol
127 preference behavior. Male F_1 legacy flies, mated to naïve females produced offspring
128 with an ethanol preference (Fig. S2a). Additionally, 14-16 day old F_1 flies displayed an
129 ethanol preference, demonstrating that F_1 flies do not have an ethanol preference decay
130 curve similar to that of the F_0 (Fig. S2b).

131

132 **Transcriptional changes**

133 Global transcriptional changes in the female head across generations were examined with
134 RNA sequencing. Heads from the F_1 and F_2 generation were collected and compared
135 with the F_0 generation, which was previously reported (Bozler et al., 2017). Analysis of
136 the F_0 data detected 98 differentially expressed transcripts (15 down and 83 up) (Fig. S3,
137 Table S4). F_1 and F_2 heads showed very few differentially expressed transcripts, 4 and 5
138 transcripts respectively. Of the differentially expressed transcripts, no transcript was

shared between groups. These data indicate that although wasp exposure itself results in global transcriptional changes in the female head, this observation does not hold true for the subsequent generations.

Germline caspases are necessary

Mid-oogenesis germline apoptosis (stage 7-8 oocytes) is triggered upon wasp exposure (Fig. 2a) (Kacsoh, Bozler, & Bosco, 2018; Kacsoh et al., 2018; Kacsoh, Bozler, Ramaswami, & Bosco, 2015). However, this wasp response is not heritable like the ethanol preference behavior, and F_1 females do not exhibit germline apoptosis (Fig. 2a). Nevertheless, maternal germline knockdown of effector caspases *Dcp-1* and *drice* produce offspring without an ethanol preference, regardless of parental treatment (Fig. 2c). Although protein-starvation triggers germline apoptosis similar to wasp exposure (Fig. 2b), offspring from mothers with starvation-induced apoptosis do not inherit an ethanol preference (Fig. 2d). This indicates that germline apoptosis in and of itself is not sufficient for inheritance of ethanol preference.

NPF and its receptor modulate germline apoptosis

NPF is known to play a role in food seeking, ethanol consumption, and numerous other reward pathways, and NPF levels decrease in the fan shaped body of female brains following wasp exposure (Kacsoh, Lynch, Mortimer, & Schlenke, 2013b). Even in the presence of wasp overexpression of NPF inhibits ethanol preference, while in the absence of wasp knockdown of NPF is sufficient to induce the ethanol preference behavior (Fig. 3a). Given this NPF modulation of ethanol preference in females we asked whether NPF

also signaled to germline cells, triggering caspases and apoptosis. Strikingly, NPF knockdown induces mid-oogenesis apoptosis in the absence of wasps (Fig. 3b), while overexpression of NPF results in no elevation in germline apoptosis even in the presence of wasps (Fig. 3b). Similarly, NPF-receptor (NPFR) knockdown alone leads to significantly elevated levels of apoptosis (28%, when compared to parental line controls $p = 6.2e^{-4}$ & $1.5e^{-4}$), and this effect is enhanced with wasp exposure (61%, $p = 7.0e^{-4}$) (Fig. 3c). Taken together these observations link ethanol preference behavior and mid-oogenesis apoptosis in the F_0 females, both processes likely caused by changes in NPF and NPFR signaling.

Changes in NPF trigger transgenerational inheritance of ethanol preference

The NPF-triggered changes in F_0 behavior and germline also correlate with observed changes in offspring. F_1 flies from mothers with NPF knockdown exhibit ethanol preference, even in the absence of wasp exposure (Fig. 3d). Inherited ethanol preference is enhanced when the parental NPF knockdown flies are exposed to wasps (Fig. 3d). By contrast, NPF overexpression in F_0 mothers exposed to wasp produced offspring lacking the ethanol preference (Fig. 3d). NPFR knockdown experiments mirror these findings: Maternal NPFR knockdown produces offspring with an ethanol preference compared to unexposed control lines, again this effect is enhanced when NPFR knockdown is paired with wasp exposure (Fig. 3e). Interestingly, overexpression of NPF in F_1 flies blocks ethanol preference in the exposed F_1 legacy group (Fig. 3f), raising the possibility that F_1 legacy flies inherit NPF in a repressed or low expression state.

We therefore speculated that regulation or depression of NPF might be a means of this behavioral inheritance. Global changes in NPF RNA were not detected in either the F_0 or F_1 female heads (Fig. S4). However, antibody staining allowed for a region specific examination of NPF protein levels (Fig. 4a). Anti-NPF signal has clear overlap with the NPF-Gal4 driving the cd8-GFP reporter (Fig. 4a). The fan shaped body has previously been implicated in ethanol preference, and therefore was a focus in this experiment(Kacsoh et al., 2013a). NPF protein levels measured through immunofluorescence were significantly reduced in the fan shaped body of F_0, F_1, and F_2 (two-generations exposed) flies (Fig. 4b). We note that NPF was not reduced in all regions of the F_1 and F_2 brains, as intensity of P1 neurons was not reduced in either the F_1 or F_2 flies, although significant reduction was observed in P1 neurons of F_0 flies (Fig. 4c).

Given the observed link between depressed NPF and oocyte apoptosis, it is notable that F_1 flies do not have germline apoptosis. It is possible that apoptosis is due to a localized decrease in NPF not shared between the two generations; perhaps the apoptosis is triggered by other NPF neurons or synapses. It is also conceivable that other neural processes are altered in the flies that we did not detect, decoupling the apoptosis and ethanol preference behaviors in the later generations.

Maternal Chromosomal Inheritance of Ethanol Preference Behavior

To determine whether maternal or paternal exposure were equally important for transgenerational inheritance of ethanol preference, wasp-exposure and mating were controlled in two separate experiments. First, mated females were exposed to wasps in the absence of male flies. Second, wasp-exposed males were mated to naïve virgin

females, removing the maternal exposure as a factor. Interestingly, F_1 offspring from exclusively maternal wasp exposure inherit ethanol preference while F_1 offspring from exclusively paternal exposures did not (Fig. 5a). We have previously reported that female flies require sight to induce a behavioral response to wasp exposure(Kacsoh et al., 2015). In further support of the maternal contribution to the inheritance of ethanol preference, blind female flies did not produce offspring with an ethanol preference (Fig. 5b). In the reciprocal experiment, blind fathers did generate ethanol-preferring offspring following a wasp exposure (Fig. 5b).

Maternal epigenetic inheritance of ethanol preference could be conferred by chromosomal elements and/or cytoplasmic factors. If ethanol preference is inherited through a chromatin mark then chromosome parental-origin tests should reveal a requirement for maternal chromosomal inheritance; however, if inheritance is conferred through cytoplasmic factors, such as noncoding-RNAs, then passage of all chromosomes through paternal gametes should have no effect since wasp-exposed females can still maternally deposit molecules and organelles into the oocyte. To test what maternal components may be conferring inheritance of ethanol preference we first focused on chromosomal elements using attached or compound chromosomes. Flies where each of the two homologs are fused cannot make haplo-chromosome gametes. Instead, they can only make gametes with one or zero copies of the fused chromosome, and therefore F1 flies inherit "pairs" of homologs that are entirely maternally or paternally derived (Fig. 5c). In this manner, we tested each of the two major autosomes for parent-of-origin effects. Using phenotypic markers, flies were sorted as having either a maternal or paternal exclusive homolog pair and assayed for ethanol preference. Chromosome-II

fusion flies had similar results when inheriting exclusively maternal or paternal Chromosome-II elements (Fig. 5c). Chromosome-III fusion flies also had inheritance of ethanol preference when receiving both copies of Chromosome-III maternally. However, flies with both copies of Chromosome-III from their fathers failed to inherit an ethanol preference (Fig. 5d). This observation has at least three implications: Most importantly, this indicates that some element on Chromosome-III must be inherited from wasp-exposed mothers in order for ethanol preference behavior to be passed on to F_1 legacy flies. This also suggests that maternal copies of the Chromosome-X, Chromosome-II or cytoplasmic factors, if important, are not sufficient for inheritance of ethanol preference. Lastly, that oocytes giving rise to eggs with zero copies of maternal Chromosome-II still confer ethanol preferences indicates that exclusion of maternal chromosomes itself does not generally interfere with transgenerational inheritance.

A Maternal NPF Locus is Required for Epigenetic Inheritance

To further delineate what parts of maternally derived Chromosome-III were required for transgenerational inheritance of ethanol preference we tested chromosomes with well defined deletions. As NPF has previously been shown to control ethanol preference behavior, we speculated that the NPF locus on Chromosome-III may be a target of maternal epigenetic reprogramming(Shohat-Ophir et al., 2012). We also observed that F_1 legacy flies inherit low levels of NPF expression specifically in the fan shaped body of the brain (Fig. 4a-b), consistent with the possibility that F_1 flies inherit repressed NPF expression. If the critical maternal Chromosome-III element is the NPF gene locus, then F_1 offspring with maternal deletions of this chromosomal region may prevent inheritance

254 of ethanol preference, much like not having inherited any maternal copies of
255 Chromosome-III (Fig. 5d). Using females with one Chromosome-III carrying a large
256 deletion of the NPF gene region and one copy of wild-type NPF on a balancer
257 Chromosome-III allowed us to ask whether an intact maternal NPF gene region was
258 necessary for F_1 inheritance of ethanol preference. We found that legacy F_1 flies from
259 unexposed mothers had no preference for ethanol, regardless of whether they inherited an
260 intact NPF gene on a balancer chromosome or a chromosomal deletion of the NPF region
261 (Fig. 5e-f). Legacy F_1 flies from exposed mothers inheriting a wild-type NPF on a
262 balancer chromosome exhibited a strong preference for ethanol, suggesting that multiple
263 rearrangements, deletions and mutations of a balancer Chromosome-III are not sufficient
264 to prevent ethanol preference in F_1 flies. By contrast, legacy F_1 flies from exposed
265 mothers inheriting a Chromosome-III deletion of the NPF gene region do not inherit any
266 preference for ethanol (Fig. 5f). This was true for two different Chromosome-III deletions
267 at the NPF locus, whereas a Chromosome-III deletion that does not disrupt the NPF gene
268 had no effect (Fig. 5f). Paternally inherited Chromosome-III deletions were not sufficient
269 to prevent ethanol preference in F_1 flies (Fig. 5f).
270

271 **Discussion**

272 Perhaps the blank slate has more written on it than we once thought. Indeed it
273 would appear that animals are bound to their ancestors in a way that some might consider
274 Lamarckian (Galloway & Etterson, 2007; Herman & Sultan, 2011; J Marshall & Uller,
275 2007). The ethanol preference we observed in this study is heritable but modifiable and
276 responsive to environmental cues, as it can be enhanced or decay across generations. Our

277 data suggest that there is an ultimate return to pre-wasp exposed state by the F_6
278 generation. If there are lingering effects of wasp exposure beyond this generation, they
279 are not detected in our assays. Not only does the ethanol preference behavior revert to
280 unexposed levels, but we also detected no priming or enhancement effect in the F_8
281 generation following a second wasp exposure (Fig S1f).

282 Inheritance of ethanol preference requires several factors: We found that the
283 initiation of the epigenetic program in the founding generation (F_0) is maternal in nature,
284 and requires effector caspases in the female germline. However, continuation of the
285 epigenetic program throughout the remaining generations is distinctly different in several
286 ways. Both male and female progeny (F_1) are able to pass on ethanol preference to their
287 offspring. Although, it is possible that the F_1 generation requires germline effector
288 caspases for the transmission of the ethanol preference, the lack of female germline
289 apoptosis and paternal ability to confer this behavior points to a caspase-independent
290 maintenance mechanism. A further and curious distinction between the generations is in
291 the ethanol preference itself, as it persists in the F_1 generation, rather than mirroring the
292 F_0 generation and decaying over 10 days.

293 The unifying mechanism behind many of these observations is the central role of
294 NPF signaling in this system. Governing both germline apoptosis and the ethanol
295 preference neuronal NPF signaling modulates the ethanol preference as well as its
296 inheritance. Maternal imprinting of the NPF locus or nearby regions has a dominant
297 effect, leading to the possibility that the F_1 paternal locus is imprinted in *trans*. It is
298 tempting to speculate on the role of canonical imprinting mechanisms, such as the

299 Polycomb repressive complexes, although a molecular apparatus remains elusive for the
300 time being.

301 This multi-generational ethanol preference underscores the importance of
302 environmental conditions on behavior and physiology. Numerous studies have indicated
303 that we may need to look beyond the individual, to longer lasting and persistent effects of
304 environmental stresses. This study illustrates the complexity of inheritance and
305 highlights the incredible resiliency and plasticity of organisms to adapt to changing
306 circumstance. Of particular interest is the conserved functions of NPF and its mammalian
307 homolog NPY in modulating a variety of human behaviors, including stress responses
308 and alcohol abuse disorders (Thorsell & Mathé, 2017). Our studies raise the intriguing
309 possibility that NPF/NPY and their receptors could be subjected to epigenetically
310 modified states determined by parental environment and experience. Germline
311 inheritance of epigenetically modified neuro-signaling networks, such as those modulated
312 by NPF/NPY, could be one mechanism through which trans-generational inheritance of
313 behavioral predispositions persist, as reported here for Drosophila. It should be noted that
314 such epigenetically inherited behaviors that persist for multiple generations could be
315 interpreted as dominant familial genetic traits. If mammalian NPY is inherited in
316 epigenetically modified states, then this would require a fundamental change in how we
317 study and view inheritance of NPY-related behavioral disorders and possible effects of
318 parental environment.

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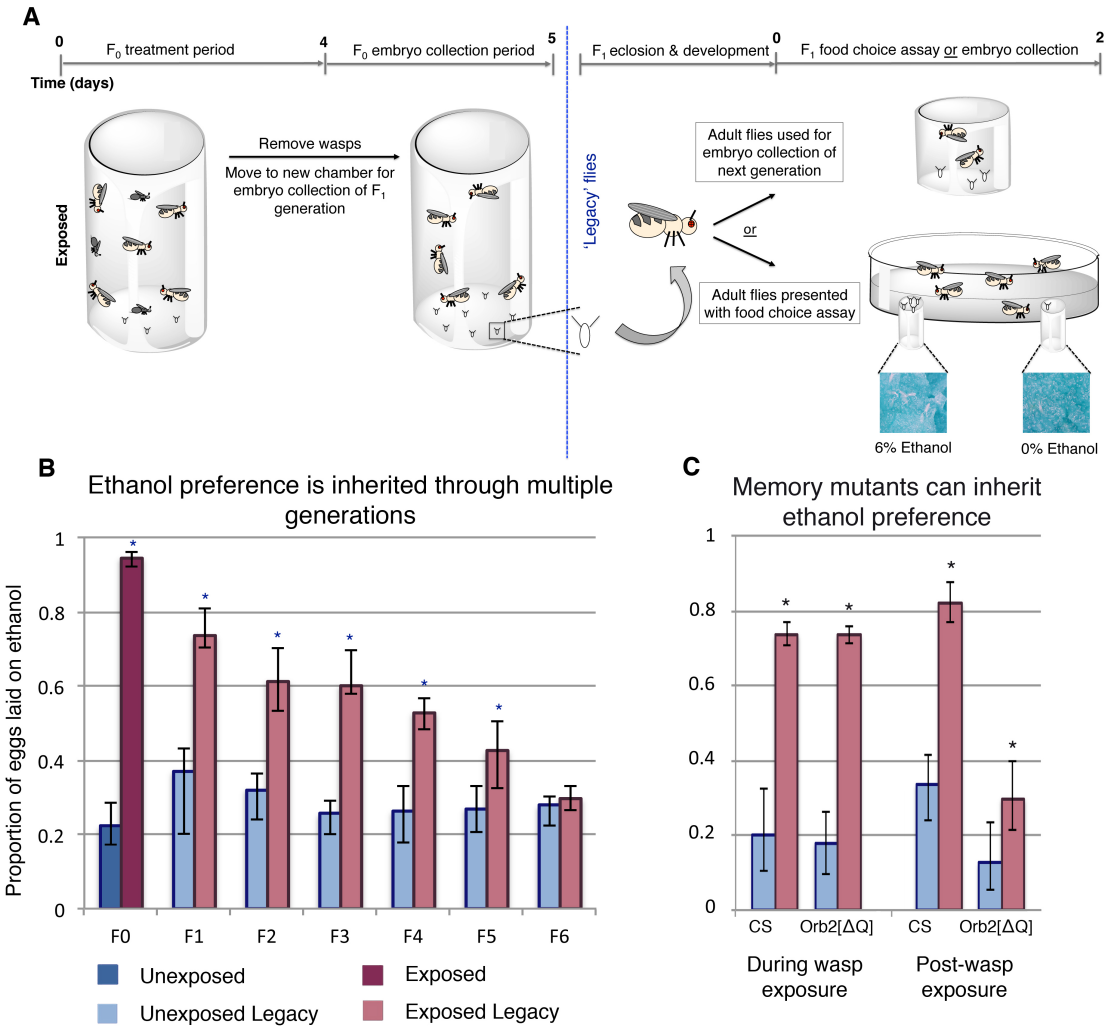

Figure 1. Maternally inherited ethanol preference persists for multiple generations.
Schematic of experimental design is shown (**A**). Flies are exposed to wasps for a period of four days prior to egg collection. The descendants from either wasp-exposed or unexposed treatment groups, termed 'legacy' flies, are reared until maturity in the absence of both wasps and parental exposure. Legacy flies are either used to propagate the next generation, or are assayed for ethanol preference. Flies from a particular generation are referred to as F_n, where n denotes the number of generations removed from the treatment. For example, the treatment group itself is F_0, whereas their direct offspring are F_1. Ethanol preference is quantified as proportion of eggs laid on ethanol food (**B**), illustrating that this behavior is heritable through the F_5 generation. Flies with deficient long-term memory were tested for transgenerational inheritance of ethanol (**C**). Asterisk indicates p-value of <0.05.

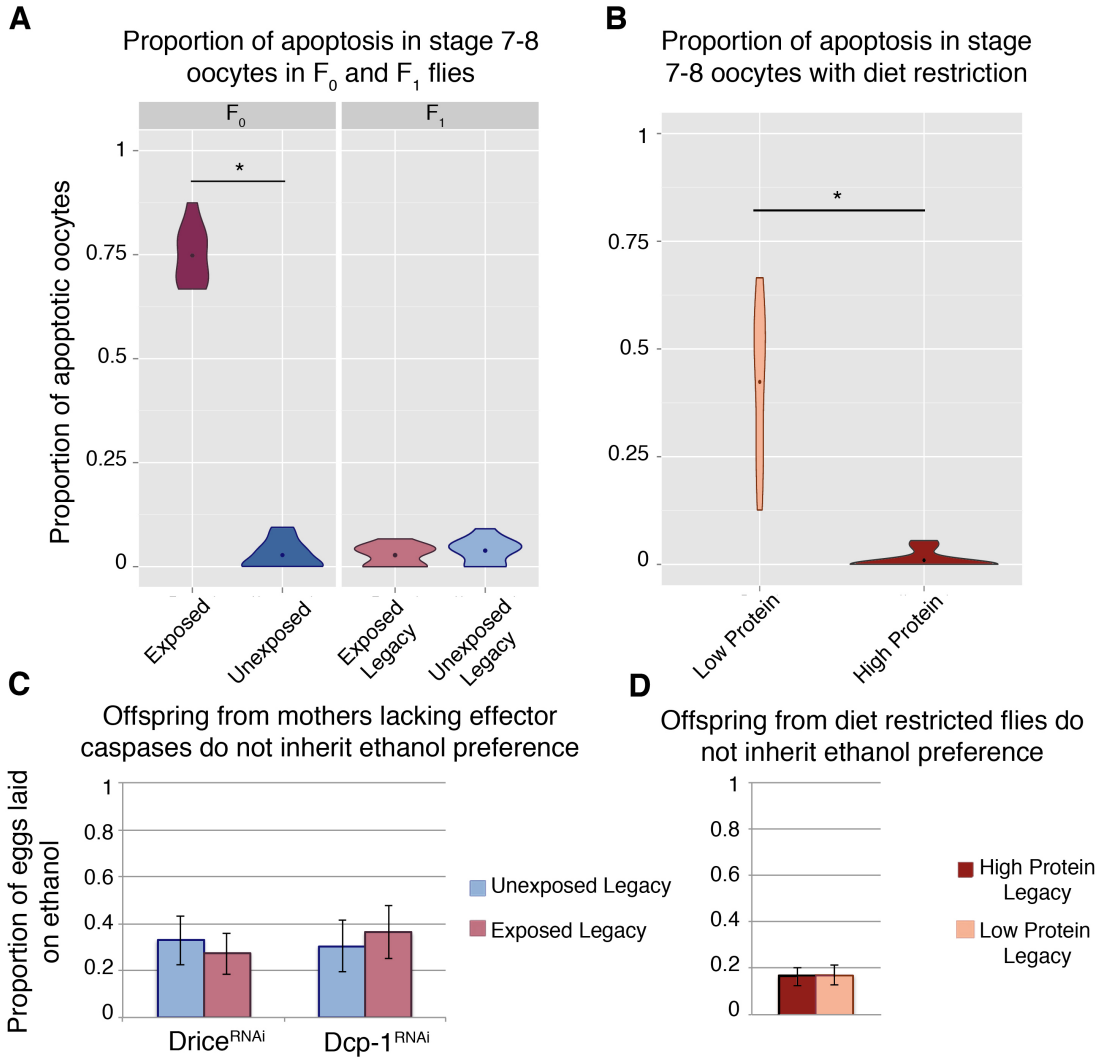

Figure 2. Germline apoptosis and activated caspases play a role in the inheritance of ethanol preference. Apoptosis in stage 7-8 egg chambers was quantified in F_0 and F_1 (legacy) flies (**A**). Flies fed a protein-restricted diet have elevated levels of stage 7-8 oocyte apoptosis (**B**). Ethanol preference is not inherited from mothers with Dcp-1 or Drice knockdown (**C**). Offspring from protein-restricted parents don't inherit an ethanol preference (**D**). Points within violin plots denote the group mean. Asterisk indicates a p-value of <0.05.

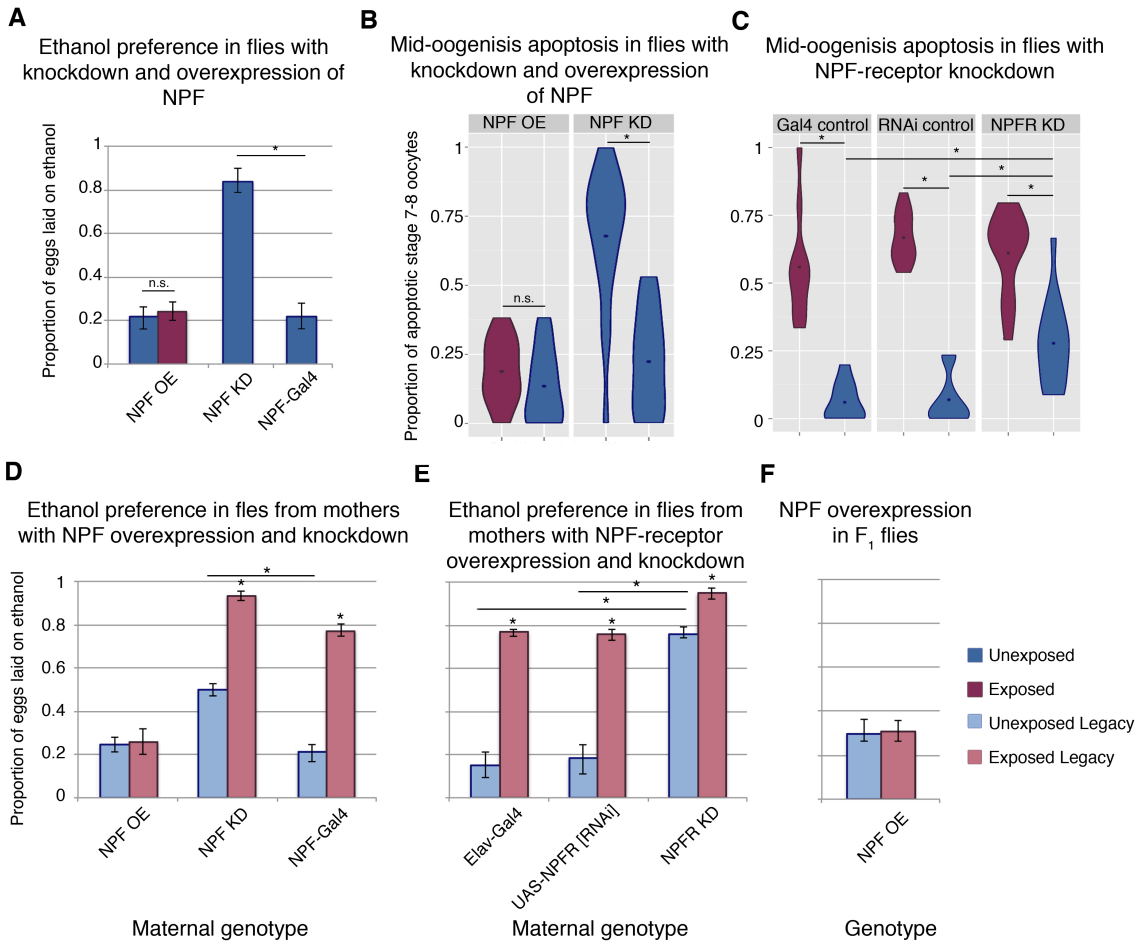

Figure 3. NPF affects ethanol preference and germline apoptosis. NPF overexpression (OE) or knockdown (KD) can alter ethanol preference (**A**). Genetic manipulation of NPF levels can alter levels of germline apoptosis (**B**). Knockdown of NPF receptor leads to increased germline apoptosis (**C**). F_1 legacy flies have altered ethanol preference depending on the maternal NPF genotype (**D**). F_1 exposed and unexposed legacy flies inherit ethanol preference from mothers with NPF receptor knockdown (**E**). F_1 legacy flies overexpressing NPF do not inherit an ethanol preference (**F**). Points within violin plots denote the group mean. Asterisk indicates a p-value of <0.05.

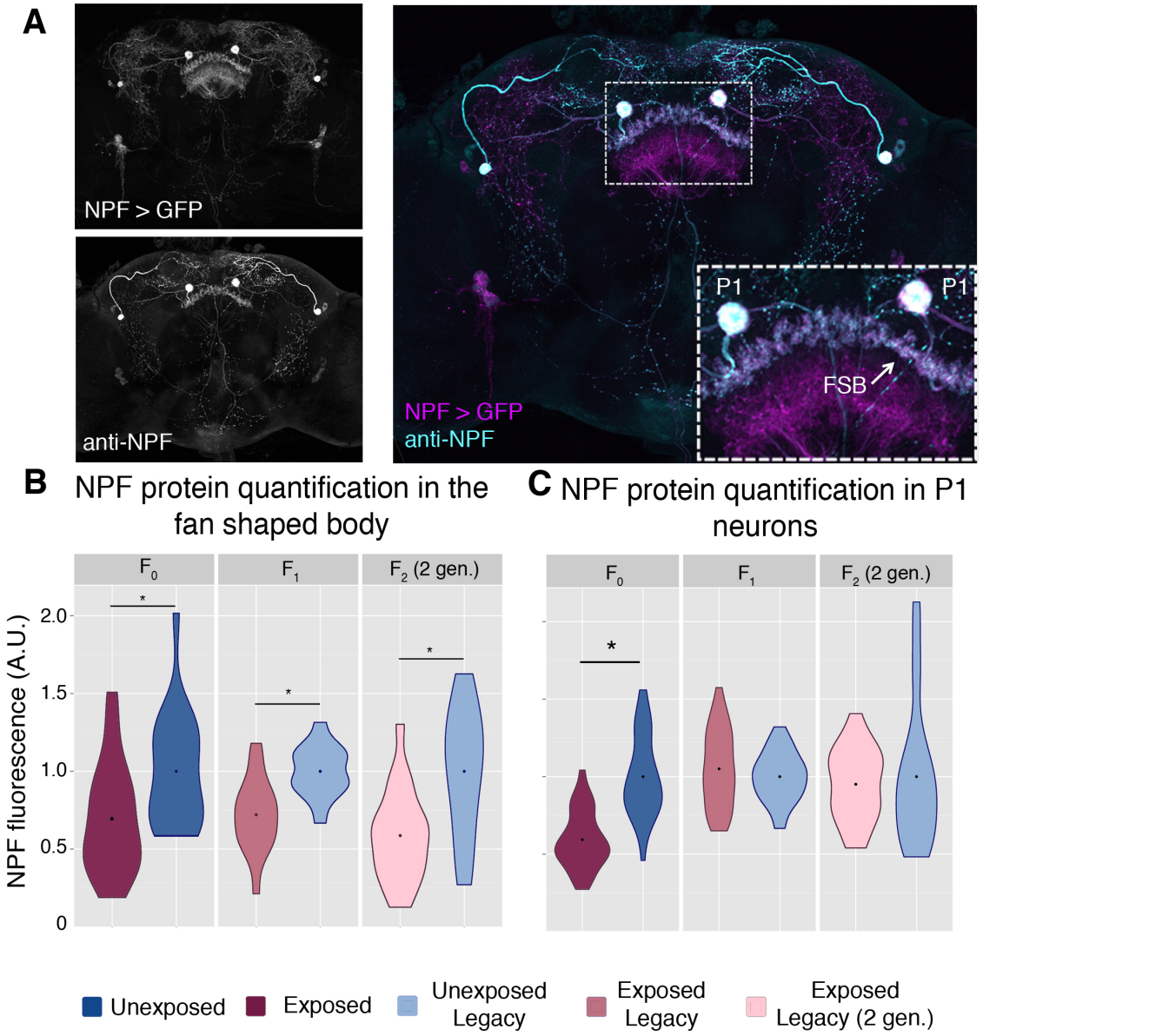

Figure 4. NPF protein is reduced in the fan shaped body following wasp exposure. NPF antibody staining has a similar pattern to that of NPF-Gal4 expression in an adult female brain, inset shows a magnification of the two large P1 neurons and the fan shaped body (FSB)(**A**). NPF protein levels are reduced in the fan shaped body across generations (**B**). NPF depression in P1 neurons is observed only in the F0 generation (**C**). Points within violin plots denote the group mean. Asterisk indicates a p-value of <0.05.

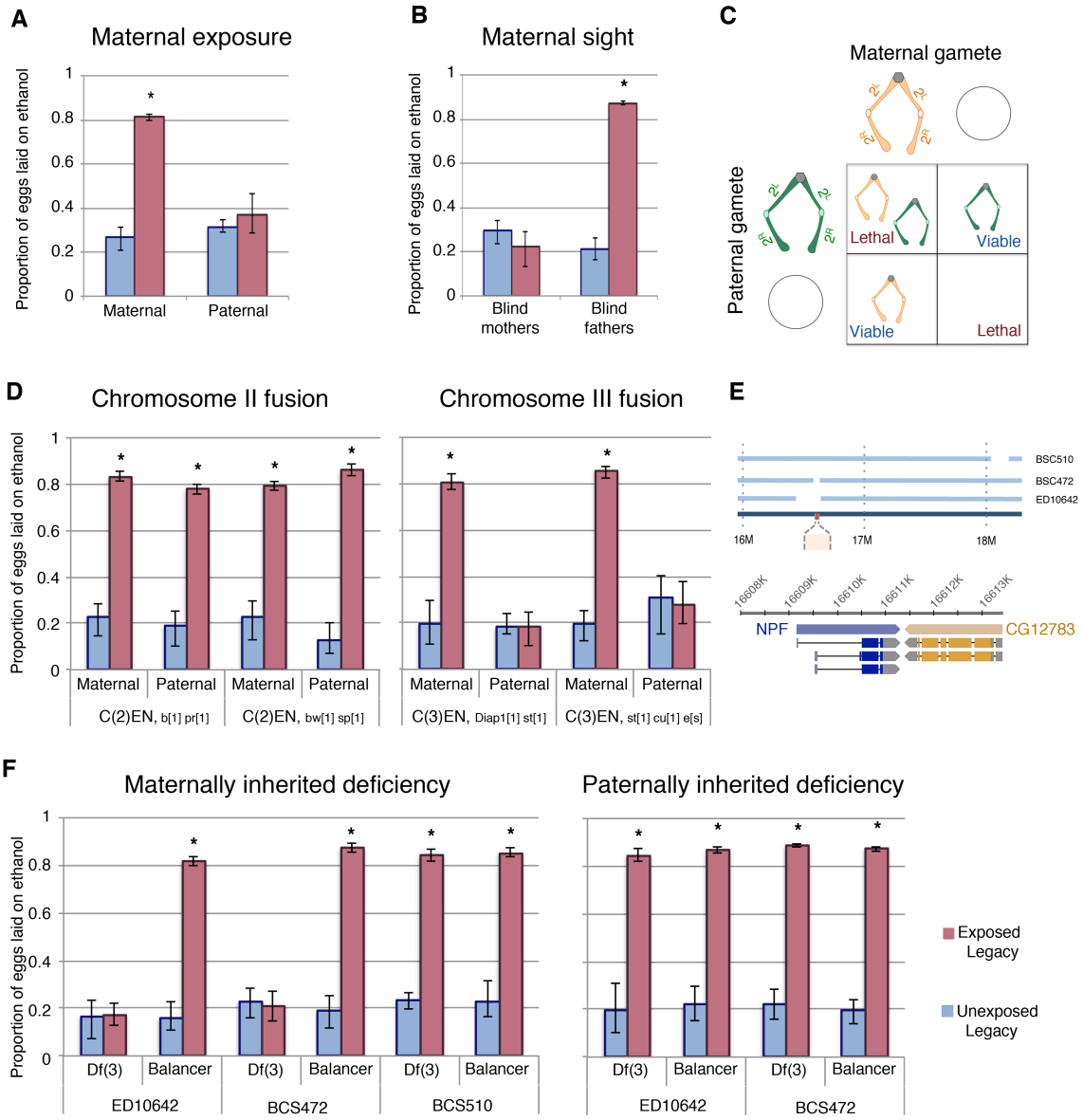

Figure 5. Maternal chromosome 3 is required for inherited ethanol preference.
Experiments with exclusively maternal or paternal wasp exposure demonstrate that maternal wasp exposure is necessary for ethanol preference inheritance (**A**). Maternal sight is required for ethanol preference inheritance, but paternal sight is dispensable (**B**). Schematic of compound chromosome 2; progeny inherit both copies from either maternal or paternal source (**C**). Flies receiving either maternal or paternal copies of chromosome 2 have inheritance of ethanol preference, but compound chromosome 3 must be maternally derived to facilitate inheritance of ethanol preference (**D**). Diagram shows the relative location of NPF (red) on chromosome 3 and the deleted region of the deficiency stock (**E**). Inheritance of ethanol preference was observed in flies receiving an intact maternal NPF locus on a balancer chromosome and not in flies from receiving a maternal NPF deficiency (Df3) chromosome: Paternal inheritance of the NPF deficiency had no effect on transmission of ethanol preference (**F**). Asterisk indicates a p-value of <0.05.

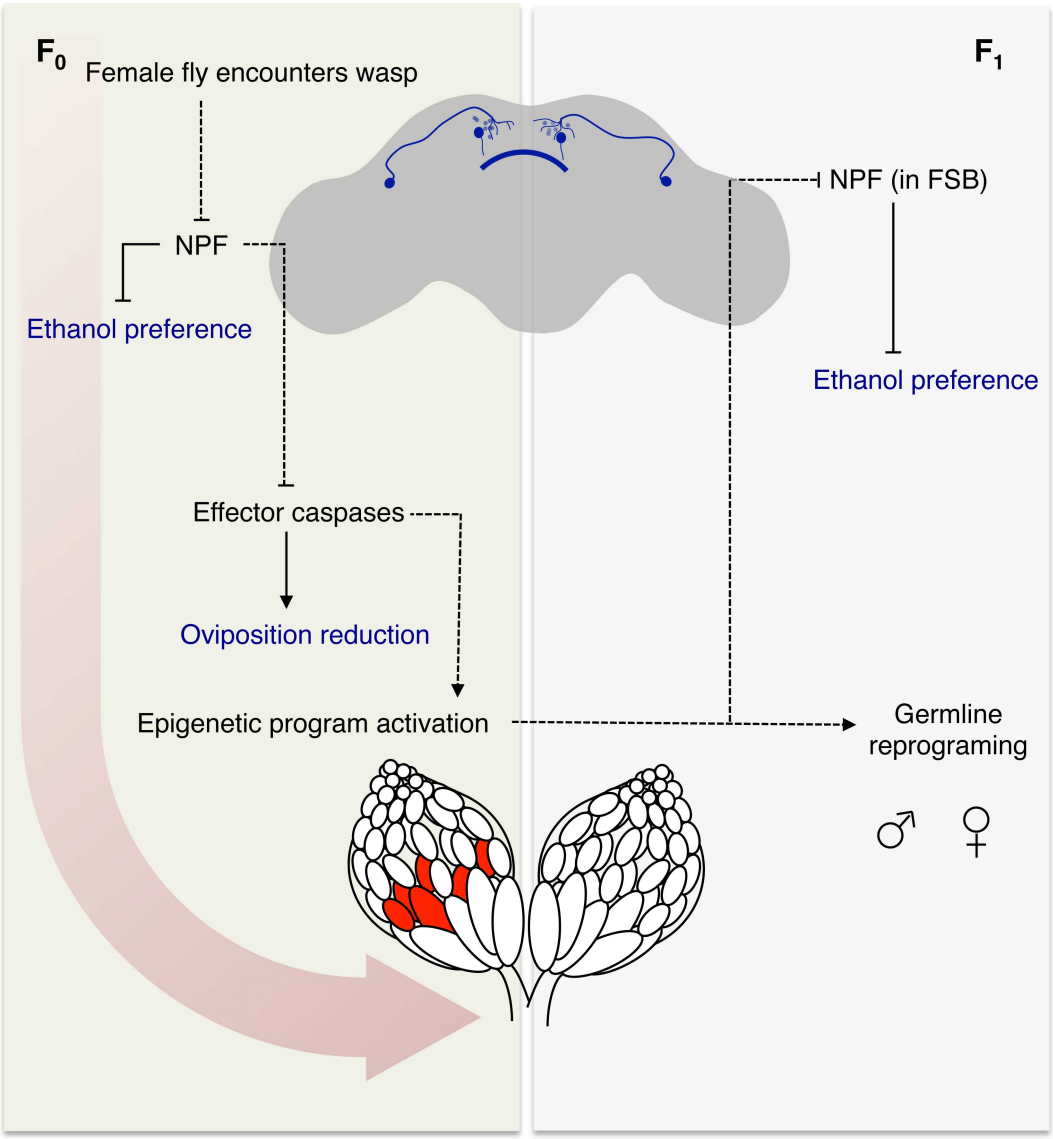

Figure 6. Model for fly-wasp mediated ethanol preference. Wasp encounter leads to a depression of NPF in the female fly brain. Normally NPF inhibits ethanol preference and caspase mediated germline apoptosis. The reduction of NPF triggers ethanol preference and germline caspases. Legacy F_1 female flies inherit depressed NPF in the FSB, both male and female progeny have altered germline. Measured behavioral outputs are in blue. Dashed lines indicate a speculative or unknown mechanism of action.

Supplementary Materials for

Transgeneratonal inheritance of ethanol preference is caused by maternal NPF repression.

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This file includes:

Materials and Methods
Figs. S1 to S4
Tables S1 to S5

Materials and Methods

Fly husbandry

Flies were maintained at room temperature on standard cornmeal-molasses media. A list of fly lines and genotypes used is reported in Table S5. Female flies were considered mature adults at three to five days post eclosion. Flies outside of this age range were not used for experimentation unless specifically noted, as for example in S Fig. 1d. Experiments involving manipulation of the maternal genotype, such as the maternal NPF knockdown, had a crossing scheme to avoid transgene expression in the F_1 generation. Virgin females with the genotype of interest were crossed to *y,w* males and offspring were scored by eye color to ensure that flies assayed were not carrying both the Gal4 and UAS constructs.

Wasp-exposure

Mature adult flies were used for wasp exposures: 40 female flies, 10 male flies, and 20 female Lh14 (*Leptopilina heterotoma*) wasps were placed in a vial with cornmeal-molasses media. This cohabitation (wasp exposure period) lasted for four days. The unexposed control consisted of the 40 female flies and 10 male flies with no wasp cohabitation. Both treatment groups were maintained at room temperature (approximately 22° C) with a 12 hour light-dark cycle for the duration of the exposure period.

At the conclusion of the exposure period, flies were separated into two cohorts. Following the removal of all wasps, one group of flies was used to propagate the next generation, while the second group was assayed for ethanol preference. Group one was

559 placed on molasses-based embryo collection plates, supplemented with yeast paste, for
560 egg collection. The collection period lasted for 24-hours, at which point the adult flies
561 were removed. First instar larvae were transferred from these embryo plates to standard
562 media vials. Larvae were density controlled to approximately 40 larvae per vial.

563 The second group was assayed for ethanol preference using a food-choice assay
564 (Kacsoh, Bozler, Hodge, Ramaswami, & Bosco, 2015). Briefly, five female flies and
565 one male fly were placed into a modified petri dish with mesh top, termed the 'fly corral'.
566 Two food sources were placed at opposite ends of the 'fly corral'. Each food source
567 consisted of 0.45 g of instant drosophila media, hydrated with 2mL liquid. Control food
568 was hydrated entirely with distilled water, where as ethanol food was prepared with
569 distilled water and a final addition of 95% ethanol to the top of the prepared food,
570 creating a food with 6% ethanol by volume. Food sources were removed and replaced
571 after 24 hours. Figures report the egg laying behavior of the first 24-hour interval unless
572 otherwise noted. Total number of eggs laid on each food source was counted in a blinded
573 fashion with treatment unknown to the counter. These egg counts are reported as a
574 proportion of eggs laid on ethanol food. Flies that encountered ethanol-containing food
575 were excluded from additional experimentation or lineage propagation. Fly corral
576 experiments had ten replicates (cages) per condition.
577
578 Transgenerational behavior experiments
579 Legacy flies, those descending from either the unexposed or exposed treatment,
580 were divided into cohorts as described above for behavioral assay or embryo collection.
581 These flies were not re-exposed to wasps except in the instance of multigenerational

582 exposure experiments. Two experiments were conducted that involved multiple
583 generations of treatment. For the successive exposures, three groups of flies were
584 assayed; exposed legacy (2 generations), exposed legacy (1 generation), and unexposed
585 legacy. In this instance, the exposed legacy (2 generations) group was generated by
586 subjecting F_1 exposed legacy flies to an additional round of wasp exposures. These flies
587 therefore had grandparental and parental wasp exposure. Exposed legacy (1 generation)
588 had parental wasp exposure only (Figure 3 B). It is important to note that the parents of
589 the 'exposed legacy (1 generation)' flies were F_1 unexposed legacy flies, and therefore
590 had the same density control and egg collection as the other groups for the
591 multigenerational duration of the experiment.

592 It is critical to note that baseline ethanol preference is highly variable depending
593 on environmental conditions. Key factors are temperature and humidity, all ethanol
594 oviposition assays were conducted in an environmentally controlled room at 25°C,
595 approximately 30% humidity (+/- 10%) with overhead lighting and a 12-hour light/dark
596 cycle. Despite these controls, baseline ethanol preference varies day-to-day. For this
597 reason, all groups for direct comparison (used in statistical tests) were tested at the same
598 time.

599 Pertaining to the nonconsecutive exposure experiments; again three groups were
600 assayed, the exposed legacy F_8 (2 generations), exposed legacy (1 generation), and the
601 unexposed legacy. For these experiments, the exposed legacy F_8 (2 generations) group
602 was created by subjecting F_7-exposed legacy flies to an additional round of wasp
603 exposures. These flies had a six-generation gap between ancestral wasp exposures. Flies

604 in the exposed legacy (1 generation) group were produced by exposing F_7 unexposed
605 legacy flies to wasps, and collecting the subsequent offspring.

606 Several experiment specific modifications were made to the methods described
607 above. To parse the maternal and paternal contributions to the inheritance of ethanol
608 preference two experiments were conducted. First, 40 mated female flies were used for
609 wasp exposure, in the absence of males. Ten males were added to the population for the
610 embryo collection period. For paternal contribution, male flies were removed from the
611 exposure chamber and mated to unexposed virgin females. To test the role of vision in
612 maternal inheritance, blind female flies mutant in ninaB, were crossed to wild type (CS)
613 males. The reciprocal experiment crossed ninaB[1] males to CS female. These
614 experiments were run in parallel and wasp exposures were preformed as previously
615 described.

616 Compound chromosome experiments crossed two fusion stocks together (either
617 chromosome-II or chromosome-III). The fusion lines retained phenotypic markers, and
618 offspring with maternal or paternal chromosomes were sorted accordingly. Deficiency
619 lines were crossed to CS flies and the genotype of the offspring (balancer or deficiency)
620 was inferred from phenotypic markers.

621 Particular modifications for the $Orb2^{\Delta Q}$ memory-mutant experiments included an
622 extra day of embryo collection. Following three-days of wasp exposure, flies and wasps
623 were moved to the embryo collection chamber for the final treatment day. Eggs were
624 collected for 24-hours in the presence of the 20 female Lh14 wasps. At the end of this
625 period, wasps were removed and a new embryo collection plate was introduced for the
626 second day of embryo collections. This second day of collection corresponds to the

627 standard embryo collection timeframe in the above-described experiments. F_1 flies had
628 the same genotype as the parental line.

629 Sibling cohorts were collected to assess the longevity of the germline change.
630 'Brood 1' flies were collected in the 24-hours immediately following the removal of the
631 wasps. 'Brood 2' flies were collected from the same parents, 10 days after the
632 termination of the wasp exposure.

633 Finally, diet restriction experiments had two groups one with high protein and the
634 other low protein diets. Low protein flies were maintained on molasses based embryo
635 plates. The high protein group was maintained in similar fashion, but with the addition of
636 yeast paste. High/low diet was maintained for four days prior to embryo collection.

637

638 Apoptosis quantification

639 Following the treatment period, ovaries were dissected and fixed in 4%
640 formaldehyde for 30 minutes. Samples were stained with DAPI and apoptosis was
641 scored based on the morphology of the nurse cell DNA. A researcher blinded to the
642 genotype and treatment group of the samples preformed the scoring. At a minimum, 15
643 ovaries were scored across 3 replicates (independent wasp exposures) for each group.

644

645 Immunostaining and microscopy

646 Antibody to neuropeptide F was generated in a rabbit to the full length NPF
647 peptide: C-Ahx-SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFamide. The
648 antibody was subsequently purified using a truncated peptide containing the first 28
649 amino acids of NPF. Following purification, the antibody was depleted using a peptide

650 of the eight amino acid C-terminal tail, shared by many neuropeptides. All peptide
651 synthesis, antigen injection, serum preparation and peptide purification and depletions
652 were performed by 21st Century Biochemicals.

653 Whole flies were fixed in 4% formaldehyde overnight at 4° C. Female brains
654 were dissected, blocked, and incubated with anti-NPF (1:1000) overnight at 4° C.
655 Antibody solution was removed and samples were blocked before the addition of the
656 secondary antibody, anti-rabbit 488 (1:200), at room temperature for two-hours. Samples
657 were counter stained with DAPI.

658 For NPF quantification, flies expressing a RFP tagged histone were dissected
659 along with treatment groups and stained in the same solution. Pixel intensity of the fan
660 shaped body (FSB) was measured in Image J. The FSB was outlined by hand and
661 intensity measured. A background measure was made of the region immediately ventral
662 to the FSB, with the same total area as the outlined FSB. The background value was
663 subtracted from FSB measurement. Finally, the background-adjusted intensity value for
664 each brain was divided by the arc length of its' FSB. This process was repeated for each
665 treatment group and the corresponding histone-RFP flies. These values were normalized
666 to the histone-RFP flies to serve as a control for batch specific variation in staining. Each
667 treatment group was normalized to the unexposed average of that replicate using the
668 formula(s):

669 $Fluorescence = (FSB_{intensity}\text{-}background_{intensity})/FSB_{length}$

670 $BatchNormalized=(Fluorescence_{CantonS}/\ Fluorescence[avg]_{his\text{-}RFP})$

671 $AFU=BatchNormalized_{exposed}/BatchNormalized[avg]_{unexposed}$

672

673 Standard fluorescent images were visualized with the Nikon Eclipse E800
674 microscope and the Olympus DP71 camera. For each experiment, wasp exposure and
675 staining were performed on two separate occasions and final data was pooled after
676 checking for the absence of a batch effect. A minimum of 10 brains were dissected for
677 each treatment replicate as well as RFP-histone co-staining brains. Final quantified
678 sample size range from 15 to 20 (normalized brains), due to sample loss or damage.
679 Imaged samples were only excluded if clear damage or trauma (from dissection or
680 staining process) was evident in the region of interest (FSB or P1 nuerons).
681
682 RNA quantification
683 Mature female flies were anesthetized with CO_2 and collected in 15 mL conical tubes,
684 either immediately following the treatment period (F_0), or 3-5 days post eclosion (F_1-F_2).
685 Flies were frozen in liquid nitrogen and briefly vortexed to separate whole heads.
686 Approximately 100 heads were collected for each replicate. A miRNeasy Kit (Qiagen)
687 with on-column DNase treatment was used for RNA isolation. Four samples of each
688 treatment group were prepared.
689 RNA samples were depleted of rRNA followed by random priming. Minimum
690 sequencing depth per sample was 40 million paired-end reads on the Illumina platform.
691 Sequencing reads were indexed to transcripts using Kallisto and the Ensembl genome
692 (BDGP6) with 100 bootstraps (Aken et al., 2016; Bray, Pimentel, Melsted, & Pachter,
693 2016). Downstream processing and statistical analyses used Sleuth (Pimentel, Bray,
694 Puente, Melsted, & Pachter, 2017). Heat maps were generated using hierarchical
695 clustering and the R package pheatmap.

696 NPF transcript was measured by qPCR (SYBR Green, Thermo-Fisher 4309155).
697 NPF primer targeted mRNA (TCCTGGTTGCCTGTGTGG,
698 TCAGCCATAGTGTTGACATCG). Actin served as the control gene
699 (CGCAAGGATCTGTATGCCAA, ACGGAGTACTTGCGCTCTGG). Fold change was
700 calculated using the delta-delta Ct method.
701
702 Statistics
703 Statistical tests were run in R (3.0.2 version, 'Frisbee Sailing'). P-values for egg count
704 data, NPF staining, and apoptosis quantification, were produced by applying a Mann-
705 Whitney Rank Sum test. Error bars presented in the egg count ethanol preference graphs
706 are bootstrap confidence intervals, generated using the boot package.
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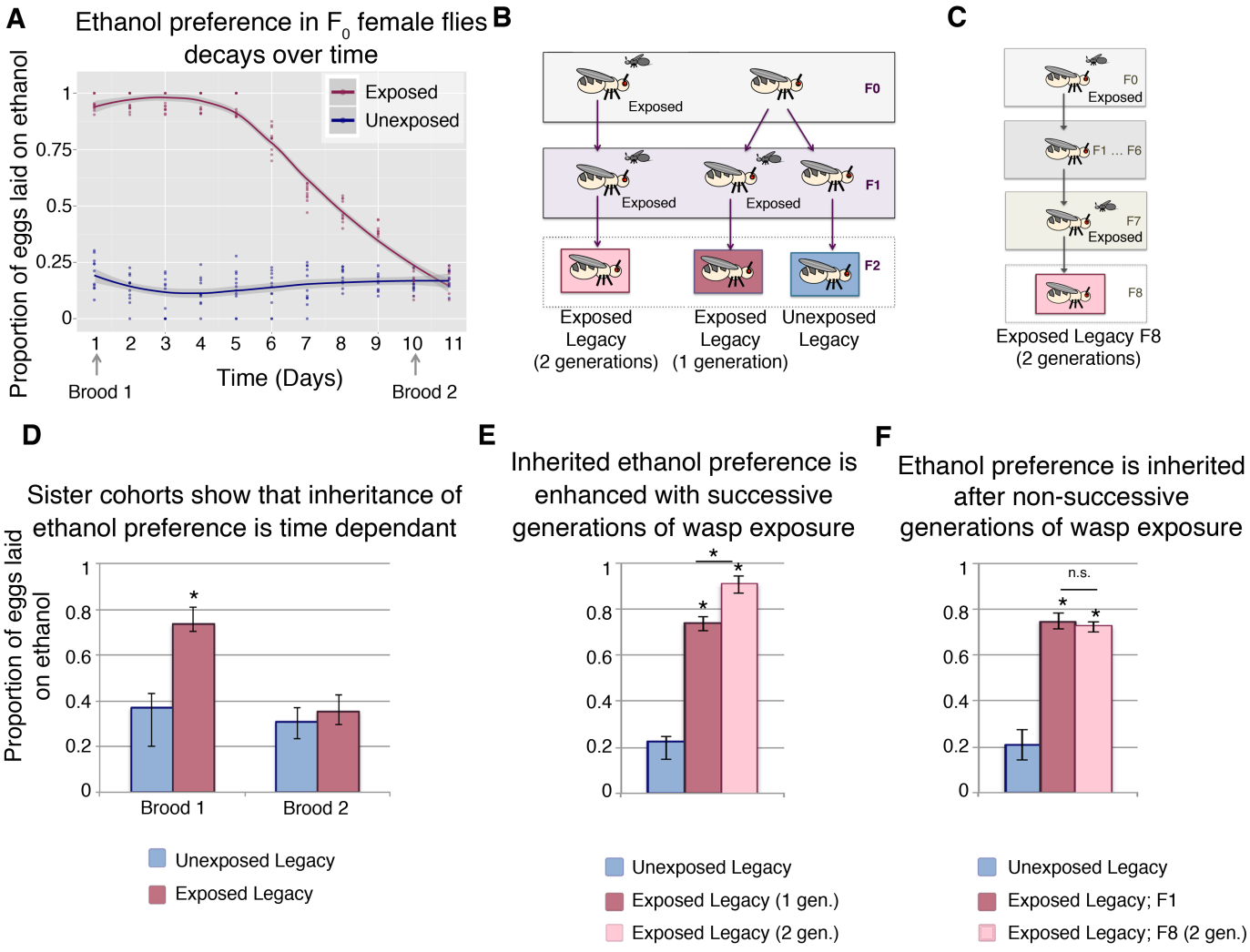

Figure S1. Temporal dynamics of wasp exposure effect inheritance of ethanol preference. Ethanol preference decays following wasp exposure (in F_0 flies), with loess regression, shaded region indicates standard error (**A**). Diagram of multigenerational exposure is shown for successive generations (**B**), and non-consecutive generations (**C**). Quantification of ethanol preference from sister cohorts collected at different intervals post-wasp exposure (**D**). Flies with successive generations of wasp exposure have enhanced ethanol preference (**E**). Alternatively, flies from a second generation of non-consecutive wasp exposure (exposure of F_7 flies) exhibit an ethanol preference similar to that of one-generation wasp exposed flies (**F**). Asterisk indicates a p-value of <0.05.

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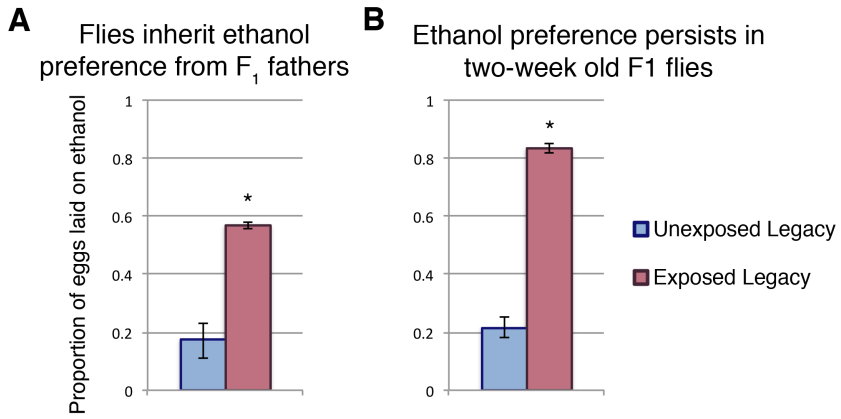


729

730 **Figure S2.** F_1 ethanol preference has distinct characteristics from those of the parental F_0
731 generation. Male F_1 flies are able to pass on ethanol preference to their offspring (**A**). Ethanol
732 preference of F_1 flies has not decayed two-weeks post eclosion (**B**). Asterisk indicates a p-value
733 of <0.05.
734

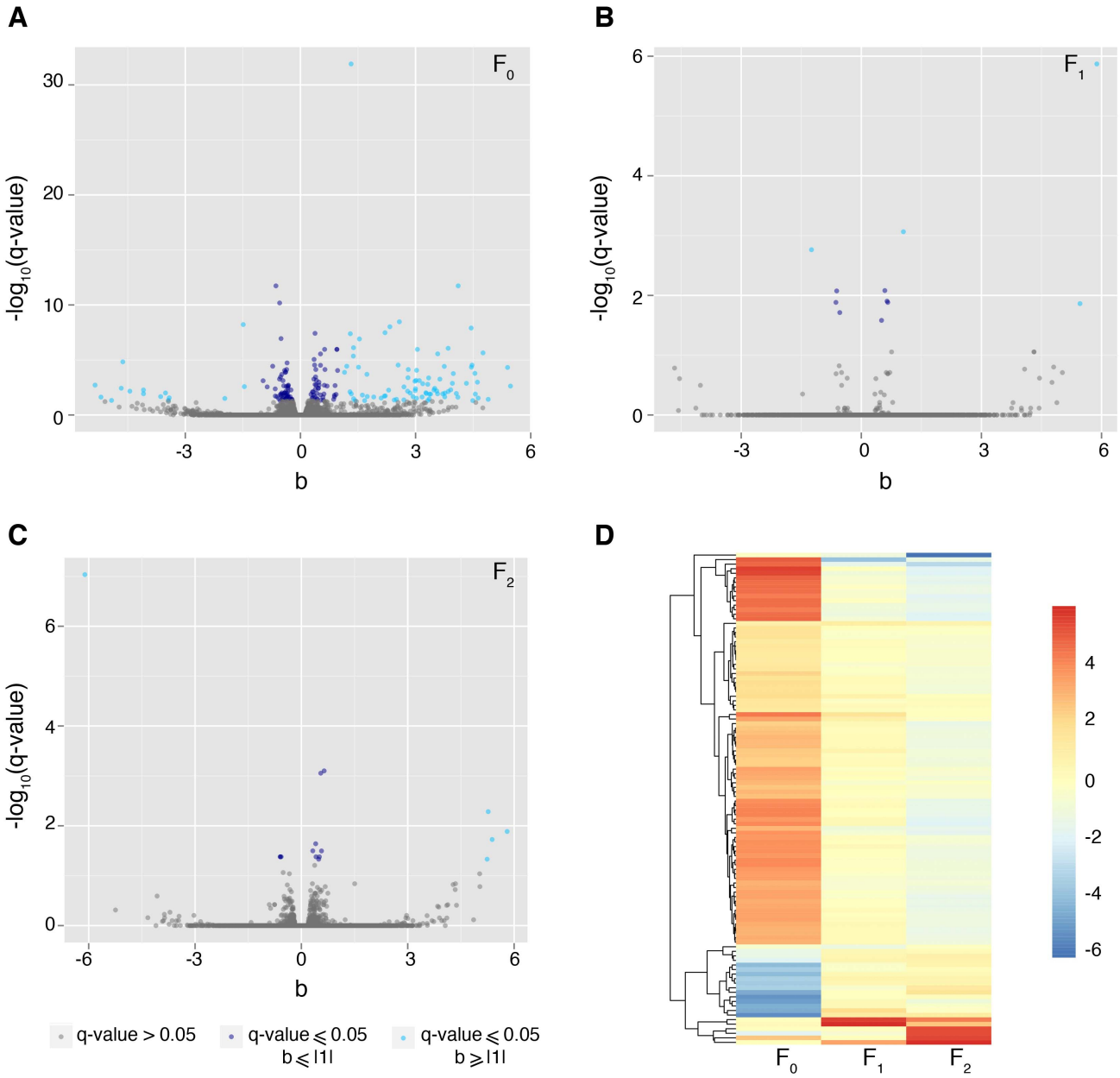
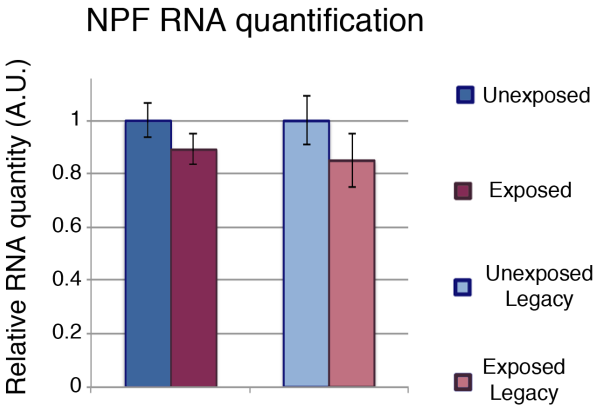


Figure S3. Global transcriptional changes in the female head. RNA sequencing was preformed on heads of F_0, F_1, and F_2 females. Volcano plots show the distribution of transcript expression and significance. F_0 flies have a considerable number of differentially expressed transcripts (**A**). Where as F_1 and F_2 heads have very few changes in transcripts (**B**) & (**C**). The beta value is approximately analogous to the natural log fold change of the transcript, and the q-value is the measure of significance. Grey points indicate a transcript with non-significant q-value, dark blue points indicate transcripts with significant q-value but that do not meet the beta value threshold. Light blue dots have significant q-value and an absolve value of beta greater or equal to one. Heat map shows the trend of transcript expression over the three generations (**D**). Transcript meeting the threshold criteria (q-value and beta) for any one generation was included in the map.



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749

750 **Figure S4.** mRNA quantification of NPF in female fly heads. Asterisk indicates a p-
751 value of <0.05.

752

753 **Table S1**. Statistical tests and p-values relating to main text figures.

Figure	Comparison groups	p-value	statistical test
1B	F0 (Exposed vs unexposed)	1.08E-05	Mann-Whitney Rank Sum
1B	F1 (Exposed vs unexposed)	8.64E-07	Mann-Whitney Rank Sum
1B	F2 (Exposed vs unexposed)	2.58E-08	Mann-Whitney Rank Sum
1B	F3 (Exposed vs unexposed)	1.29E-08	Mann-Whitney Rank Sum
1B	F4 (Exposed vs unexposed)	4.13E-06	Mann-Whitney Rank Sum
1B	F5 (Exposed vs unexposed)	0.00833	Mann-Whitney Rank Sum
1B	F6 (Exposed vs unexposed)	0.6063	Mann-Whitney Rank Sum
1C	CS-during (Exposed vs unexposed)	0.0001817	Mann-Whitney Rank Sum
1C	Orb2-during (Exposed vs unexposed)	0.000278	Mann-Whitney Rank Sum
1C	CS-post (Exposed vs unexposed)	1.08E-05	Mann-Whitney Rank Sum
1C	Orb2-post (Exposed vs unexposed)	0.02065	Mann-Whitney Rank Sum
2A	F0 (Exposed vs unexposed)	0.0001593	Mann-Whitney Rank Sum
2A	F1 (Exposed vs unexposed)	0.3144	Mann-Whitney Rank Sum
2B	High protein vs low protein	0.0001079	Mann-Whitney Rank Sum
2C	Drice[RNAi] (Exposed vs unexposed)	0.5787	Mann-Whitney Rank Sum
2C	Dcp-1[RNAi] (Exposed vs unexposed)	0.05889	Mann-Whitney Rank Sum
2D	High protein vs low protein	0.933864	Mann-Whitney Rank Sum
3A	NPF OE (Exposed vs unexposed)	0.5787	Mann-Whitney Rank Sum
3A	NPF KD vs NPF-Gal4	1.08E-05	Mann-Whitney Rank Sum
3B	NPF OE (Exposed vs unexposed)	0.1758	Mann-Whitney Rank Sum
3B	NPF KD vs NPF-Gal4	1.76E-05	Mann-Whitney Rank Sum
3C	Elav-Gal4 (Exposed vs unexposed)	0.0001697	Mann-Whitney Rank Sum
3C	NPFR[RNAi] (Exposed vs unexposed)	3.07E-06	Mann-Whitney Rank Sum
3C	NPFR KD (Exposed vs unexposed)	0.0007069	Mann-Whitney Rank Sum
3C	NPFR KD vs NPFR[RNAi] unexposed	0.0001503	Mann-Whitney Rank Sum
3C	NPFR KD vs Elav-Gal4 unexpoed	0.0006232	Mann-Whitney Rank Sum
3D	NPF OE (Exposed vs unexposed)	0.4359	Mann-Whitney Rank Sum
3D	NPF KD (Exposed vs unexposed)	1.08E-05	Mann-Whitney Rank Sum
3D	NPF-Gal4 (Exposed vs unexposed)	1.08E-05	Mann-Whitney Rank Sum
3D	NPF KD vs NPF-Gal4 unexposed	1.08E-05	Mann-Whitney Rank Sum
3E	Elav-Gal4 (Exposed vs unexposed)	0.0001817	Mann-Whitney Rank Sum
3E	NPFR[RNAi] (Exposed vs unexposed)	0.0001817	Mann-Whitney Rank Sum
3E	NPFR KD (Exposed vs unexposed)	0.0001817	Mann-Whitney Rank Sum
3E	NPFR KD vs NPFR[RNAi] unexposed	1.08E-05	Mann-Whitney Rank Sum
3E	NPFR KD vs Elav-Gal4 unexpoed	0.0001817	Mann-Whitney Rank Sum
3F	NPF OE (Exposed vs unexposed)	0.7333	Mann-Whitney Rank Sum
4B	F0 (Exposed vs unexposed)	0.009027	Mann-Whitney Rank Sum
4B	F1 (Exposed vs unexposed)	0.0004949	Mann-Whitney Rank Sum
4B	F2 (Exposed vs unexposed)	0.002572	Mann-Whitney Rank Sum
4C	F0 (Exposed vs unexposed)	3.09E-11	Mann-Whitney Rank Sum
4C	F1 (Exposed vs unexposed)	0.3972	Mann-Whitney Rank Sum
4C	F2 (Exposed vs unexposed)	0.6378	Mann-Whitney Rank Sum
5A	maternal (Exposed vs unexposed)	0.000011	Mann-Whitney Rank Sum
5A	paternal (Exposed vs unexposed)	0.1904	Mann-Whitney Rank Sum
5B	Blind mothers (Exposed vs unexposed)	0.3154	Mann-Whitney Rank Sum
5B	Blind fathers (Exposed vs unexposed)	0.0002712	Mann-Whitney Rank Sum
5D	Chr-II Maternal C(2)EN b[1] pr[1] (Exposed vs unexpos	0.0001796	Mann-Whitney Rank Sum
5D	Chr-II Paternal C(2)EN b[1] pr[1] (Exposed vs unexpose	0.0002695	Mann-Whitney Rank Sum

5D	Chr-II Maternal C(2)EN bw[1] sp[1] (Exposed vs unexpo	0.0004456	Mann-Whitney Rank Sum
5D	Chr-II Paternal C(2)EN bw[1] sp[1] (Exposed vs unexpo	0.0002695	Mann-Whitney Rank Sum
5D	Chr-III Maternal C(3)EN Diap1[1] sp[1] (Exposed vs unexposed)	0.0006306	Mann-Whitney Rank Sum
5D	Chr-III Paternal C(3)EN Diap1[1] sp[1] (Exposed vs unexposed)	0.7308	Mann-Whitney Rank Sum
5D	Chr-III Maternal C(3)EN st[1] cu[1] e[s] (Exposed vs unexposed)	0.0006258	Mann-Whitney Rank Sum
5D	Chr-III Paternal C(3)EN st[1] cu[1] e[s](Exposed vs unex	0.8857	Mann-Whitney Rank Sum
5F	Maternal Df(3)ED10642 (Exposed vs unexposed)	0.8796	Mann-Whitney Rank Sum
5F	Maternal ED10642-balancer (Exposed vs unexposed)	0.0001766	Mann-Whitney Rank Sum
5F	Maternal Df(3)BCS472 (Exposed vs unexposed)	0.7569	Mann-Whitney Rank Sum
5F	Maternal BCS472-balancer (Exposed vs unexposed)	0.000278	Mann-Whitney Rank Sum
5F	Maternal Df(3)BCS510 (Exposed vs unexposed)	0.002141	Mann-Whitney Rank Sum
5F	Maternal BCS510-balancer (Exposed vs unexposed)	0.002141	Mann-Whitney Rank Sum
5F	Paternal Df(3)ED10642 (Exposed vs unexposed)	0.0001756	Mann-Whitney Rank Sum
5F	Paternal ED10642-balancer (Exposed vs unexposed)	0.0001796	Mann-Whitney Rank Sum
5F	Paternal Df(3)BCS472 (Exposed vs unexposed)	0.0004426	Mann-Whitney Rank Sum
5F	Paternal BCS472-balancer (Exposed vs unexposed)	0.0002451	Mann-Whitney Rank Sum
S1D	Brood 1 (Exposed vs unexposed)	2.49E-10	Mann-Whitney Rank Sum
S1D	Brood 2 (Exposed vs unexposed)	0.6305	Mann-Whitney Rank Sum
S1E	Exposed (1 gen) vs unexposed	1.08E-05	Mann-Whitney Rank Sum
S1E	Exposed (2 gen) vs unexposed	1.08E-05	Mann-Whitney Rank Sum
S1E	Exposed (1 gen) vs exposed (2 gen)	1.08E-05	Mann-Whitney Rank Sum
S1F	Exposed (1 gen) vs unexposed	1.82E-04	Mann-Whitney Rank Sum
S1F	Exposed F8 (2 gen) vs unexposed	1.08E-05	Mann-Whitney Rank Sum
S1F	Exposed (1 gen) vs exposed F8 (2 gen)	0.472	Mann-Whitney Rank Sum
S2A	Exposed vs unexposed	1.08E-05	Mann-Whitney Rank Sum
S2B	Exposed vs unexposed	0.000181	Mann-Whitney Rank Sum
S4	F0 (Exposed vs unexposed)	0.3429	Mann-Whitney Rank Sum
S4	F1 (Exposed vs unexposed)	0.3429	Mann-Whitney Rank Sum

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Table S2. Oregon R experimental data. Key experiments were replicated using the additional wild-type strain OreR. "Corresponding Figure" indicates the experiment that was replicated: A listing of Fig1B therefore indicates that the experimental conditions for Figure 1B were duplicated using OreR flies.

		Day 1			Day 2		
Corresponding Figu for Duplicate Experime	**Description**	**Mean (experimenta**	**Mean (control**	**p-value**	**Mean (experimenta**	**Mean (control)**	**p-value**
1B	F0	0.913	0.288	1.08E-05	0.927	0.276	1.08E-05
1B	F1	0.704	0.303	1.08E-05	0.695	0.276	1.81E-04
1B	F2	0.679	0.286	1.08E-05	0.691	0.271	1.82E-04
1B	F3	0.675	0.25	1.08E-05	0.647	0.266	1.82E-04
1B	F4	0.556	0.211	2.44E-04	0.534	0.221	1.08E-05
1B	F5	0.366	0.277	0.07526	0.41	0.221	0.000129
1B	F6	0.273	0.263	0.7394	0.224	0.238	0.6305
Not shown in figure	F7	0.202	0.208	0.4359	0.211	0.193	0.3429
2A	F0 apoptosis (Exposed vs unexpose	0.705	1.70E-02	0.000144			
2A	F1 apoptosis (Exposed vs unexpose	0.017	3.10E-02	0.2931			
5A	Paternal	0.36	0.45	0.1655	0.34	0.35	0.8534
S1D	Brood 2	0.23	0.27	0.705	0.24	0.27	0.6842
S1E	Exposed (1 gen) vs unexposed	0.744	0.213	1.81E-04	0.723	0.186	1.82E-04
S1E	Exposed (2 gen) vs unexposed	0.91	-	1.08E-05	0.924	-	1.82E-04
S1E	Exposed (1 gen) vs exposed (2 gen	-	-	1.81E-04	-	-	1.81E-04
S1F	Exposed (1 gen) vs unexposed	0.79	0.258	1.82E-04	0.81	0.223	1.82E-04
S1F	Exposed F8 (2 gen) vs unexposed	0.76	-	1.82E-04	0.792	-	1.80E-04
S1F	Exposed (1 gen) vs exposed F8 (2 g	-	-	0.1209	-	-	0.7048

763 **Table S3**. Canton S day-2 data; mean(s) and p-value(s).
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Corresponding Figur	Description	Mean (experimental)	Mean (control)	p-value
1B	F0	0.959	0.201	2.71E-04
1B	F1	0.8	0.369	3.38E-06
1B	F2	0.689	0.349	1.29E-08
1B	F3	0.595	0.258	6.12E-06
1B	F4	0.523	0.262	2.58E-08
1B	F5	0.431	0.267	0.01256
1B	F6	0.237	0.264	0.1577
Not shown in figure	F7	0.158	0.163	0.7674
2C	Drice[RNAi]	0.295	0.291	0.6774
2C	Dcp-1[RNAi]	0.329	0.261	0.4359
2D	Low protein v high (control)	0.163	0.173	0.575213
5A	maternal	0.754	0.244	0.000182
5A	paternal	0.425	0.29	0.001031
S1D	Brood 1	0.81	0.335	3.12E-08
S1D	Brood 2	0.33	0.273	0.1903
S1E	Exposed (1 gen) v Unexposed	0.769	0.164	1.08E-05
S1E	Exposed (2 gen) v Exposed (1 gen)	0.908	-	1.82E-04
S1E	Exposed (2 gen) v Unexposed	-	-	1.82E-04
S1F	Exposed (1 gen) v Unexposed	0.737	0.172	1.82E-04
S1F	Exposed F8 (2 gen) v Exposed (1 ge	0.784	-	0.08873
S1F	Exposed F8 (2 gen) v Unexposed	-	-	1.82E-04
S2A	paternal (F1)	0.569	194	1.08E-05
S2B	Two-week old F1	0.833	0.127	0.000022

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767 **Table S4**. RNA sequencing results from female fly heads.

Transcript ID	Gene Name	q-value	b	Data set
FBtr0332131	CG42255	1.25E-32	1.320266165	F0
FBtr0087004	Amy-p	1.84E-12	4.110027459	F0
FBtr0086983	Amy-d	3.40E-09	2.577135928	F0
FBtr0077220	lcs	6.05E-09	-1.490020237	F0
FBtr0074375	CG15865	9.56E-09	2.330606904	F0
FBtr0302527	CG33346	1.25E-08	4.447797727	F0
FBtr0079607	Mur29B	3.32E-08	2.203753518	F0
FBtr0083372	CG8907	4.13E-08	1.300100455	F0
FBtr0079136	CG11029	1.23E-07	1.536917077	F0
FBtr0078953	Skp2	7.62E-07	1.383516598	F0
FBtr0076063	Muc68E	8.69E-07	3.848174764	F0
FBtr0085472	CG7567	1.09E-06	3.047754006	F0
FBtr0074626	CG15043	2.25E-06	4.756372803	F0
FBtr0083164	CG5399	2.78E-06	3.575384524	F0
FBtr0088432	Def	4.45E-06	1.378119365	F0
FBtr0310455	CG43680	1.48E-05	-4.62766656	F0
FBtr0083823	CG4783	2.91E-05	2.545384042	F0
FBtr0300506	CG42397	2.91E-05	4.470662763	F0
FBtr0083030	Mf	3.72E-05	1.206382413	F0
FBtr0087792	CG13321	3.85E-05	3.747204916	F0
FBtr0083026	CG3987	4.51E-05	1.512498647	F0
FBtr0087797	CG13323	4.51E-05	4.453581565	F0
FBtr0345521	Amy-p	4.80E-05	5.396352897	F0
FBtr0308229	CG13810	7.37E-05	2.80924618	F0
FBtr0310431	CG32633	8.70E-05	3.026437806	F0
FBtr0306289	CG43236	0.000133305	1.148856362	F0
FBtr0087796	CG13324	0.000164657	4.546214478	F0
FBtr0075069	CG6839	0.00017266	3.983281993	F0
FBtr0079500	Acp1	0.000202054	1.732372175	F0
FBtr0100028	obst-H	0.000407687	2.801483865	F0
FBtr0072879	CG13806	0.000427043	3.340054966	F0
FBtr0084957	Kaz-m1	0.000550781	3.632660457	F0
FBtr0076119	Muc68D	0.000716285	3.009816876	F0
FBtr0072121	CG3906	0.000755348	3.097285784	F0
FBtr0078908	CG14645	0.000861022	2.982720707	F0
FBtr0088161	alphaTry	0.001040886	4.610885525	F0
FBtr0072938	CG1246	0.001327451	4.323284994	F0
FBtr0302854	Phae2	0.001328434	3.958342039	F0
FBtr0080370	Oatp33Eb	0.001447556	3.186358438	F0
FBtr0081865	CG11672	0.001634501	3.317037455	F0
FBtr0346383	whe	0.001932977	-5.350102624	F0
FBtr0076044	CG14125	0.002352177	5.47281471	F0
FBtr0082900	CG33109	0.002647488	-1.460634359	F0
FBtr0112526	CG34324	0.002778253	2.696535128	F0
FBtr0306805	CG9626	0.003698463	-4.667810078	F0
FBtr0290275	tgy	0.00374776	3.498024408	F0
FBtr0114524	Pgant4	0.004076224	2.997652431	F0
FBtr0303096	Scsalpha	1.35E-06	5.882649017	F1
FBtr0083763	CG31221	0.000862597	1.050809868	F1
FBtr0100880	mt:ND4L	0.001724056	-1.24427752	F1

FBtr0077465	ed	0.013747666	5.462236135	F1
FBtr0089084	Eph	9.25E-08	-6.107714474	F2
FBtr0084901	CG5028	0.005199103	5.269815553	F2
FBtr0076263	simj	0.012996463	5.803771619	F2
FBtr0074393	CG5162	0.018783539	5.381305094	F2
FBtr0304571	RyR	0.046679175	5.239173713	F2

Table S5. Drosophila stock list and source information.

name	genotype	source	stock numbe
CS	+	Bosco Lab	
OreR	+	Bosco Lab	
Orb2[deltaQ]	Orb2[deltaQ]	Bosco Lab	
NPF-Gal4	y[1] w[*]; P{w[+mC]=NPF-GAL4.1}2	Bloomington Stock C	25681
UAS-NPF	UAS-NPF	Shen Lab	
Elav-Gal4	Elav-Gal4	Bosco Lab	
UAS-NPF[RNAi]	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02555}attP2	Bloomington Stock C	27237
UAS-NPFR[RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01959}attP2	Bloomington Stock C	25939
UAS-Dcp1[RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM05120}attP2	Bloomington Stock C	28909
UAS-Drice[RNAi	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00398}attP2	Bloomington Stock C	32403
Matα-Gal4	w[*]; P{w[+mC]=matalpha4-GAL-VP16}V37	Bloomington Stock C	7063
ninaB	w[*]/Dp(1;Y)y[+]; ninaB[1], P{w[+mC]=UAS-ninaB.G}3	Bloomington Stock C	24776
compound ch-II	C(2)EN, b[1] pr[1]	Bloomington Stock C	1112
compound ch-II	C(2)EN, bw[1] sp[1]	Bloomington Stock C	1020
compound ch-III	C(3)EN, Diap1[1] st[1]	Bloomington Stock C	1114
compound ch-III	C(3)EN, st[1] cu[1] e[s]	Bloomington Stock C	1117
Df(3)10642	w[1118]; Df(3R)ED10642, P{3'.RS5+3.3'}ED10642/TM6C, Sb[1]	Bloomington Stock C	9482
Df(3)BSC472	w[1118]; Df(3R)BSC472/TM6C, Sb[1] cu[1]	Bloomington Stock C	24976
Df(3)BSC510	w[1118]; Df(3R)BSC510/TM6C, Sb[1] cu[1]	Bloomington Stock C	25014
yw	y[1]w[1]	Bloomington Stock C	1495