Antimicrobial peptides do not directly contribute to aging in *Drosophila*, but improve lifespan by preventing dysbiosis

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Abstract

Antimicrobial peptides (AMPs) are innate immune effectors first studied for their role in host defense against bacterial and fungal infections. Recent studies have implicated these peptides in the clearance of aberrant cells and various neurological processes including neurodegenerative syndromes. In *Drosophila*, an array of AMPs are produced downstream of the Toll and Imd NF-κB pathways in response to infection. Many studies have suggested a role for the Imd pathway and AMPs in aging in this insect, supported by the upregulation of AMPs with aging (so-called "inflammaging"). However, functional studies using RNAi or over-expression have been inconclusive on whether and how these immune effectors impact aging.

Leveraging a new set of single and compound AMP gene deletions in a controlled genetic background, we have investigated how AMPs contribute to aging. Overall, we found no major effect of individual AMPs on lifespan, with a possible exception of *Defensin*. However, *ΔAMP14* flies lacking 14 AMP genes from seven families display a reduced lifespan. Interestingly, we found an increased bacterial load in the food medium of aged *ΔAMP14* flies, suggesting that the lifespan reduction of these flies was due to a failure in controlling the microbiome. Consistent with this idea, use of germ-free conditions extends the lifespan of *ΔAMP14* flies. Overall, our results do not point to an overt role of individual AMPs in lifespan. Instead, we find that AMPs collectively impact lifespan by preventing dysbiosis over aging. This is consistent with previous studies that have shown that AMPs control the gut microbiome, and that dysbiosis is detrimental upon aging. In the course of our experiments, we also uncovered a strong impact of a cryptic *Drosophila nora virus* infection on lifespan that should be taken into consideration in aging studies.

Introduction

Antimicrobial peptides (AMPs) are innate immune effectors found in plants and animals that show microbicidal activity in vitro. They are typically cationic and amphipathic, and disrupt microbial membranes that are more negatively charged [1–4]. Recent studies in various animal models have pointed to a role for AMPs beyond microbial infection, notably in aging and aging-related diseases [5–12]. Like other animals, AMP genes are upregulated upon aging in *Drosophila*, and disparate studies have suggested a role for AMPs as causative agents of aging, neurodegeneration or mitochondrial stress [7,8]. Here we have leveraged a set of isogenic AMP deficient flies to analyze the contribution of AMPs to aging in *Drosophila*.

Antimicrobial peptides are well characterized in *Drosophila* for their contribution to host defence. There are currently eight families of inducible AMPs known in *D. melanogaster*: the antifungals *Drosomycin*, *Baramicin*, and *Metchnikowin*; *Cecropins* (four inducible genes) and *Defensin* that have both antibacterial and some antifungal activities; and *Drosocin*, *Attacins* (four genes) and *Diptericins* (two genes) that primarily exhibit antibacterial activity [13,14]. In addition, the *Drosophila* genome encodes many other host defence peptide families such as *Daisho* (two genes) and *Bomanin* (12 genes), where overt antimicrobial activity in vitro is not yet demonstrated, but functional studies have shown they are important in vivo to resist microbial infection [15,16]. While most *Drosophila* defence peptide genes are strongly induced in the fat body in response to systemic infections, many show specific and complex patterns of expression in tissues such as the trachea, gut, salivary glands, or reproductive tracts [17,18].

AMPs genes are regulated at the transcriptional level by the Toll and Imd NF-kB signaling pathways upon systemic infection, or by the Imd pathway in local epithelia [19,20]. It is well established that Imd and Toll deficient flies show marked susceptibility to microbial infection. Until recently, the importance of immune effectors downstream of these pathways, notably AMPs, was unclear. With the advent of CRISPR gene editing, we systematically deleted seven families of AMP genes (*Defensin*, *Cecropin*, *Drosocin*, *Attacin*, *Diptericin*, *Drosomycin*, and *Metchnikowin*) and analyzed their contributions to host defence individually or collectively. We found that *Drosophila* AMPs are essential downstream of the Imd pathway to resist systemic infection by Gram-negative bacteria. *Drosophila* AMPs also contribute downstream of the Toll pathway to combat fungi and to a lesser extent Gram-positive bacterial infection, although *Bomanins* play a more prominent role against these micro-organisms [15,21,22]. Use of fly lines carrying combinations of AMP mutations revealed that they can function either additively or synergistically against some microbes, but in some cases AMPs exhibit striking specificity, with one peptide contributing most of the AMP-dependent defence against a specific pathogen [22–24]. *Drosophila* AMPs are also important to control the fly microbiome downstream of the Imd pathway, particularly for their role in regulating Gram-negative bacteria like *Acetobacter* [25].

While *Drosophila* AMPs were initially investigated for their contribution to host defence, AMP upregulation is also observed in non-immune contexts, including anti-

tumor defence [26–28], neurodegeneration [29–33], and aging [34,35]. This suggests that AMPs could have roles beyond their traditional function as microbicidal agents. Notably, transcriptomic studies revealed that high AMP expression, reflecting an increase in Imd pathway activity, is a hallmark of aging in flies [36–39]. This is reminiscent of the situation observed in humans where a low-grade chronic inflammation is observed upon aging, termed "inflammaging" [40–42]. The key question now is to decipher if this high and chronic activation of the immune response is simply a marker of aging, or if cytotoxic immune effectors like AMPs accelerate aging-associated syndromes.

To address this question, several reports have investigated the role of the Imd pathway and AMPs in aging in *Drosophila* with contrasting outcomes. Most of these studies report that Imd pathway mutants are short-lived, notably those targeting the Imd transcription factor *Relish*. However one study suggested that Imd mutation itself improves lifespan, as did fat body or whole body AMP knockdown [43]. The negative impact of Imd pathway downregulation has been associated with defects in gut homeostasis [44], exaggerating the decline in gut resilience upon aging [45]. The guts of aged *Relish* mutant flies display precocious loss of compartmentalization, increased permeability, and dysplasia [46]. Some of these symptoms could be reverted when flies were raised axenically pointing to a role of the microbiome in the precocious aging of the gut [44,46]. Another study reports that AMP genes are strongly induced in the head of old flies, and that silencing *Relish* in glia can extend life span [29]. This would suggest that Imd-mediated immune responses drive aging by directly affecting brain activity. Indeed, many studies report that suppression of the Imd pathway can prevent neurodegeneration in various neurodegenerative disease models. For instance, flies mutated in the serine/threonine kinase *ataxia telangiectasia mutated* (*ATM*, also called *tefu*) display reduced lifespan associated with increased neuronal lesions, which can be rescued by combination with *Relish* mutation. *Relish* deletion also rescues the aging-dependent neurodegeneration of *Cdk5-alpha* mutant flies [32], and interesting recent studies even showed that deletion of AMPs can improve fly survival after traumatic brain injury [33,47]. An effect of AMP overexpression in aging has also been proposed, albeit with conflicting results. Cao et al. [30] reported AMP upregulation and neurodegeneration in the brains of *dnr1* mutant flies, and showed that AMP overexpression in neurons was sufficient to induce neurodegeneration. Badinloo et al. [48] found that chronic and ubiquitous AMP overexpression reduced fly lifespan alongside induction of mitochondrial stress. These results suggest AMPs could be deleterious to host fitness, also supported by evolutionary studies showing that AMP deletions segregate in wild populations, suggesting a fitness cost of AMP maintenance [24,49,50]. However in direct contradiction to this idea, an earlier report found that excess expression of certain AMPs could extend lifespan [51].

Presently, we do not know how AMPs impact aging, and a direct link between AMPs and aging remains to be demonstrated. Some of the conflicting results mentioned above may arise from use of different conditions (e.g. temperature, sex, mating status, nutrition) [52–55], microbiome differences (perhaps explaining contradictory findings [56–58]), or lack of control over genetic background, including transgene insertions [59,60], all of which can influence longevity. In the absence of mutants, these studies

have relied on the use of over-expression or RNAi to modulate AMPs, methodologies with certain limitations [61,62]. As a result, exactly how the AMPs contribute to aging remains unclear.

Here we have leveraged a recently generated collection of AMP mutations to analyze whether these immune effectors impact aging. We find that individual AMP deletions do not markedly affect aging, with the possible exception of *Defensin*. However, *ΔAMP14* flies lacking 14 AMP genes displayed reduced lifespan associated with microbial dysbiosis. Rearing *ΔAMP14* flies in germ-free conditions significantly rescues lifespan, indicating that AMPs contribute to lifespan through their impact on the microbiome. In contrast, depleting the microbiome of *Relish* mutant flies did not rescue lifespan, suggesting the Imd pathway can affect lifespan independent of regulating AMP genes. Together, our results indicate that AMPs are likely not direct contributors to the aging process, though they have a major impact on aging through AMP-microbiome interactions. Thus, our study, using loss of function mutations, clarifies the role of these innate immune effectors in aging.

Results

Drosophila nora virus has a profound effect on the lifespan of DrosDel wild-type flies

To address the role of AMP genes in aging, we first compared the lifespan of flies lacking eight AMPs located on Chromosome II (*ΔAMP Chr2* lacking *Def, Dro, AttA,B,C, Mtk,* and *DptA,B)* to their DrosDel isogenic wild-type (*iso* w^{1118}*)* controls. Of note, all our mutations are in the DrosDel isogenic genetic background (unless mentioned otherwise), and are negative for the endosymbiont *Wolbachia*. We observed a striking effect where *ΔAMP Chr2* deficient flies displayed a marked lifespan extension compared to wild-types (Fig. 1A). This first result would be consistent with studies suggesting AMPs negatively impact lifespan. However, we were surprised by the short lifespan of our *iso* w^{1118} wild-type compared to other aging studies, and to a second wild-type included in these experiments, *Oregon R* (*OR-R* in Fig. 1A). *Drosophila* can carry a number of cryptic bacterial or viral infections that affect fitness [63]. Notably infection with *Drosophila nora virus* is common in lab stocks, and this virus has been shown to reduce lifespan [64]. We thus suspected some component of the virome/microbiome of our *iso* w^{1118} flies could be affecting our *iso* w^{1118} wild-type but not *ΔAMP Chr2* flies. We therefore cleared our flies of their virome/microbiome through bleaching and assessed the lifespan of the cleaned *iso* w^{1118} stock (protocol in materials and methods). At the same time, we screened our *iso* w^{1118} flies for a panel of common contaminating viruses. We did detect *Drosophila nora virus* (hereafter "*nora*") in our *iso* w^{1118} flies, but not in our *ΔAMP Chr2* mutants. Strikingly, the bleaching treatment markedly improved *iso* w^{1118} lifespan, which then rivalled that of *ΔAMP Chr2* flies (Fig. 1B, compared to Fig. 1A). Thus, our wild-type reference had artificially reduced lifespan, likely due to a cryptic *nora virus*

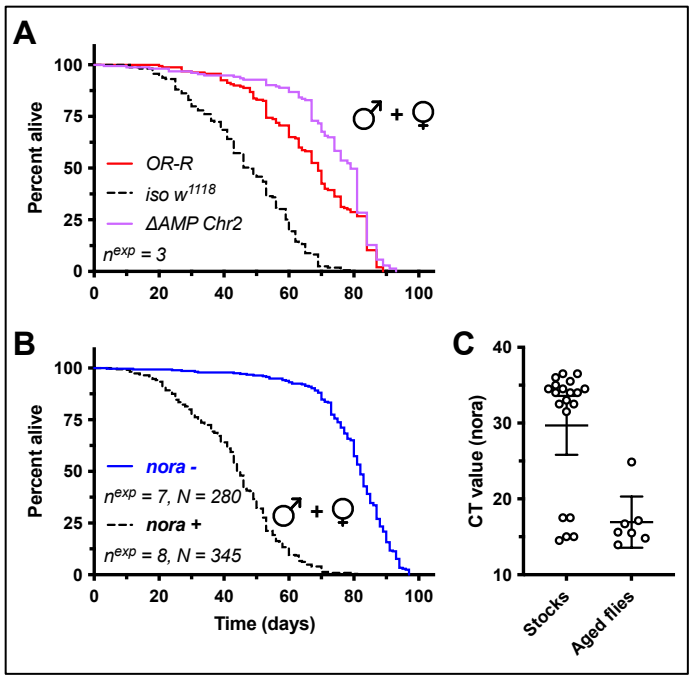

Figure 1: *Drosophila nora virus* significantly reduces lifespan of DrosDel iso w^{1118} flies. A) Comparison of lifespan from early experiments using two wild-types (*iso w^{1118}* and *OR-R*) alongside compound AMP mutants lacking *Def, Dro, AttA,B,C, Mtk,* and *DptA,B*, which are deleted in *ΔAMP Chr2* flies. B) Effect of *nora* clearance on *iso w^{1118}* lifespan. Number of independent experiments (n^{exp}) and total flies (N) is reported. C) *Nora* titres measured by CT value in 18°C source stocks (Stocks) or *nora*-positive flies aged 3+ weeks kept at densities of 20 flies per vial (Aged flies). CT values represent *nora* titre from 5ng total fly RNA per 10µL qPCR reaction.

infection. While we took care to control for genetic background effects, this stock, at the time, was not an appropriate baseline for comparison.

After detecting this contamination in our wild-type flies and seeing the impressive deleterious effect *nora* had on lifespan in our hands, we screened various lab stocks for *nora* virus. We found that 20/44 arbitrarily selected stocks (both from our lab and others) were *nora*-positive, though *nora* titres were typically found only at low levels under standard rearing conditions. We also checked RNA samples collected from aged *nora*-positive flies (aged >3 weeks), finding consistent high *nora* titres (Fig. 1C). These stocks also often showed abdominal bloating at older ages pre-empting mortality (personal observation). We thus suspect *nora virus* contamination contributed greatly to the early mortality of our *iso w^{1118}* flies in our first lifespan experiments (Fig. 1A), with a more drastic effect than previously reported [64].

Methodology for measuring lifespan and control genotypes

Following these results, we screened all our fly stocks for *nora virus* and bleached *nora*-positive stocks before use in experiments. Subsequent longevity experiments were carried out with the following conditions: we used our standard food medium (recipe in materials and methods), flipped flies three times per week, with ~20 flies per vial, using mated males or females (sexes kept separate), and performed lifespans at 25°C unless otherwise specified. As an assay for brain health, we also monitored locomotor competence during aging using the climbing pass rate assay at 5, 40, 50, and 60 days post-eclosion (dpe) unless specified otherwise.

To compare lifespan, we first used a Cox mixed model commonly used in the literature. However, we found this statistical method was overly-sensitive for our purposes, as even minor differences in lifespan were returned as highly significant ($p < .001$). This statistical significance seemed to reflect interactions across variables that were not consistent from one set of experiments to the next; i.e. significance was not

necessarily indicative of biological relevance (see File S4). Moreover, inspecting sum survival curves suggests these differences largely reflect variation around the mean *iso* w^{1118} wild-type lifespan, or at least are not especially striking. Thus, we paired our lifespan analyses with one-way ANOVA statistics run on median lifespans to get a more stringent measure of lifespan differences, with a greater value placed on consistency across experiments. We report both statistical approaches in figures, although we prefer to make inferences only for trends strong enough to show up in median lifespan analyses. As such, we use one-way ANOVA p-values by default in the text to report significant lifespan differences. Merged male and female lifespans are shown in the main figures (separated male and female lifespan summaries are provided in File S1).

Before we address the impact of AMP mutations on lifespan, we analyzed the lifespans of mutants affected in the Toll (*iso* spz^{rm7}) or Imd (*iso* Rel^{E20}) pathways that have been backcrossed in the DrosDel background [65]. We also compared our DrosDel *iso* w^{1118} $white^{-/-}$ flies with another wild-type (*OR-R*), which has $white^{+/+}$ red eyes. Of note, *white* gene deletion may affect lifespan through e.g. the role of *white* in the brain [60], or through *white* regulation of intestinal stem cell proliferation [59]. We further included non-isogenic flies with previously-reported lifespan effects to show how control genotypes behave in our conditions, and set expectations for the size of lifespan effect we could reasonably observe in our hands. This included: *methuselah* mutants suggested to have exceptionally long lifespan (mth^1) [66], a *dnr1* mutation that reduces lifespan previously associated with neurodegeneration and aberrant AMP induction ($dnr1^{2-133}$) [30], and ATM^8 flies with precocious neurodegeneration caused by autophagy defects, also associated with Imd pathway activation [31]; mth^1, $dnr1^{2-133}$, and ATM^8 were not backcrossed into the DrosDel background, and have red eyes. Our experiments confirmed that ATM^8 flies have significantly shorter lifespan (Fig. 2A,C) associated with poor climbing competence (Fig. 2D). We also observed that our *OR-R* wild type displays a shorter lifespan compared to our DrosDel *iso* w^{1118} wild-type. This result is noteworthy, as it suggests that median lifespans of different wild types readily differ by ~10 days in our hands, setting a baseline of difference that could reasonably be owed to genetic background effects. Contrary to expectation, the lifespan of mth^1 flies was not longer than *OR-R* wild-type ($p > .10$), and the lifespan of $dnr1^{2-133}$ was not significantly reduced ($p > .10$) compared to *OR-R* wild-type at 25°C (Fig. 2A,C). However, we did observe improved climbing competence of mth^1 flies into old age (Fig. 2D), which suggests these flies do have a form of improved fitness with aging in our hands. These experiments also revealed that Rel^{E20} flies and to a lesser extent spz^{rm7} flies have reduced lifespan, suggesting a contribution of the Toll and Imd pathways to aging.

We also repeated these lifespan experiments at 29°C, which represents a more stressful temperature for *Drosophila melanogaster*, causing more rapid aging [52]. When we reared flies at 29°C, as expected, we observed precocious aging both in terms of lifespan and climbing pass rate compared to 25°C for all genotypes tested: *iso* w^{1118}, *OR-R*, $dnr1^{2-133}$, mth^1, and Rel^{E20} (Fig S1A,B). Again, mth^1 flies retained climbing

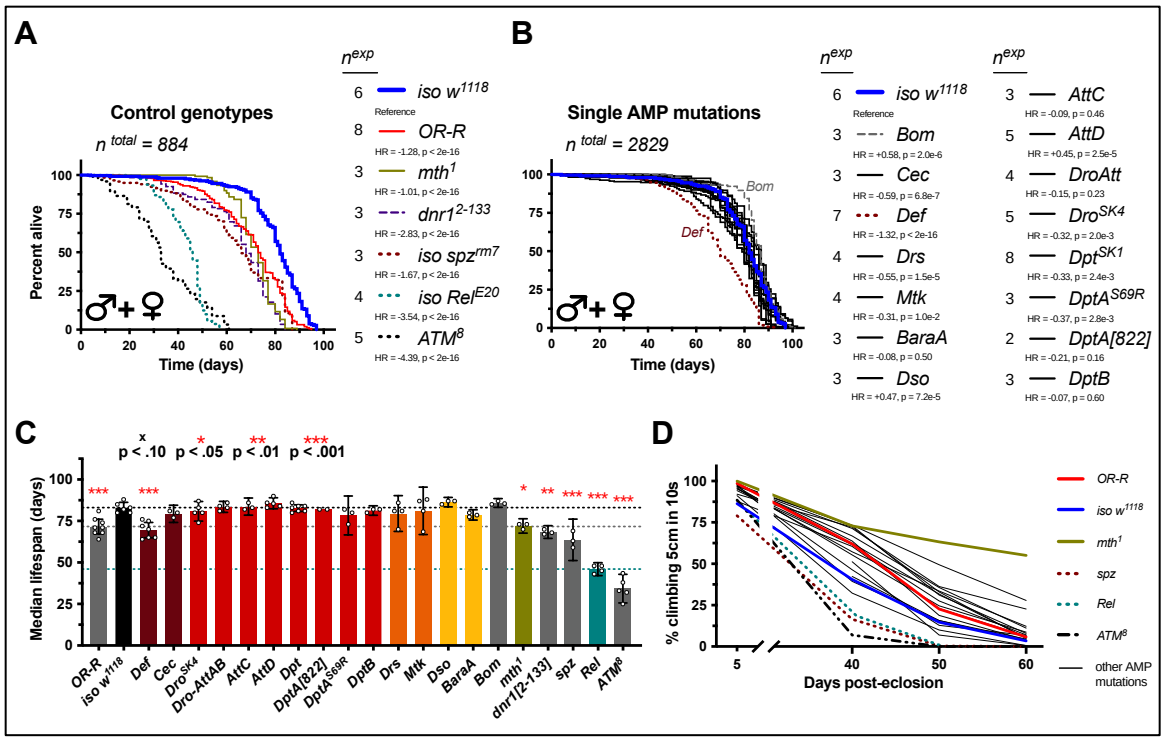

Figure 2: Individual AMP gene deletions do not drastically affect lifespan. A) Cumulative lifespans of male and female flies with various genetic backgrounds. B) Cumulative lifespans of single gene AMP mutants. Most AMP mutant lifespans (black lines) cluster around the wild-type (blue line), except *Def*^{SK3}. *Bom*$^{\Delta 55C}$ is also noted as an outlier perhaps living slightly longer than *iso w*^{1118}. C) Median lifespans where each data point represents one replicate experiment (cumulative of 20 males and 20 females). Median lifespans analysis suggests that the only AMP mutation significantly differing from *iso w*^{1118} was *Def*^{SK3}. Of note, the impact of *Def* on lifespan was not corroborated using *Defensin RNAi* (Fig. S3). *Bom*$^{\Delta 55C}$ median lifespans were not different from *iso w*^{1118}. Horizontal dotted lines indicate median lifespans of *iso w*^{1118} (top), *OR-R* (middle), or *Rel*^{E20} (bottom). Statistic summaries (p-values: ˣ, *, **, ***) reflect comparisons to *iso w*^{1118}. D) Climbing pass rates suggest most AMP mutants climb like wild-type flies, while *methuselah* mutants uniquely retain climbing competence into old age (see Fig. S3 for detailed analysis).

competence longer than other genotypes (Fig. S1C), despite no lifespan extension effect. Of note, differences in lifespan between *OR-R* and *iso w*^{1118} were lost when flies were raised at 29°C. The previous *dnr1* mutant study showing reduced lifespan used 29°C [30], and *dnr1*$^{2\text{-}133}$ had reduced lifespan compared to *OR-R* at 29°C ($p < .05$) but not DrosDel *iso w*^{1118} ($p > .10$). Ultimately, we found the *dnr1* lifespan effect to be mild in our hands, but with a consistent trend at both 25°C and 29°C compared to the wild-type flies used here.

Collectively, we observed that, *ATM*, *Relish* and to a lesser extent *spz* mutation reduced lifespan consistent with previous studies [29,31,67]. The impact of *dnr1* was less marked than previously published, which could rely in part on the wild-type used for comparison: Cao et al. [30] used *Canton S*, while here we compare lifespans to *OR-R* and DrosDel *iso w*^{1118}. Surprisingly, we did not observe lifespan extension in *methuselah* mutants. However, *mth*^{1} flies retain climbing competence far better than any other genotype assayed, indicating these flies do have a form of improved aging in our hands.

Single AMP gene deletion does not drastically affect lifespan

We next analysed the lifespan of flies lacking individual or small genomic clusters of AMP genes at 25°C. This included mutants for all 'classical' AMPs from the seven gene families initially identified: *Def*, *Cec* (removing 4 *Cecropins*), *Dro* alone, and also *Dro* alongside *AttA* and *AttB*, and then *AttC*, *AttD*, *Mtk*, *Drs*, *DptA* and *DptB* together or individually, as well as mutants for the more recently described Toll-regulated effector genes *Daisho (Dso1* and *Dso2)*, *Baramicin A (BaraA)*, and *Bomanins (Bom$^{\Delta 55C}$*, removing ten *Bom* genes at cytogenetic map 55C*)*.

We found no major effect on lifespan of any AMP mutation individually, as survival curves tended to disperse randomly around the *iso w^{1118}* lifespan curve, and median lifespans were not significantly different from *iso w^{1118}* (Fig. 2B-C). The only exception to this trend was *Def^{SK3}* flies, which had noticeably reduced lifespan compared to *iso w^{1118}* ($p < .001$). In general, climbing competence was also distributed in a wild-type range comparable to *iso w^{1118}* or *OR-R* flies, and none were exceptionally good climbers into old age like *mth^{1}* flies (Fig. 2D), though *Mtk* and *Drs* mutants had slightly improved climbing into old age (Fig. S3). The *Def^{SK3}* reduction in lifespan is striking, but we note that its lifespan is still in a wild-type range comparable to *OR-R* flies (median lifespans: *iso w^{1118}* = 83.0, *OR-R* = 73.5, *Def^{SK3}* = 69.6, and *OR-R* vs. *Def^{SK3}* $p > .10$). Although our lines are isogenized over seven rounds of recombination, we cannot rule out that a locus close to the *Defensin* gene on the *Def^{SK3}* chromosome could affect lifespan; i.e. an effect unrelated to *Defensin* directly (see File S4). When we tried to validate this effect using ubiquitous (*Actin5C-Gal4*) or glia-specific (*Repo-Gal4*) *Def* interfering RNA (*Def-IR*), we saw no effect of *Def-IR* compared to controls (Fig. S3). Thus we could not support this *Def^{SK3}* mutation effect with RNA interference (RNAi). During these RNAi experiments, we also noted that lifespan differed markedly according to the RNAi or GAL4 background used (Fig. S2), similar to the ~10 day lifespan differences we observed comparing *OR-R* and *iso w^{1118}* (Fig. 2A).

Collectively, our study using isogenic mutants shows that individual AMP mutations do not significantly affect lifespan, with the possible exception of *Def^{SK3}*. Overall we conclude that deleting single AMP genes has no effect on lifespan beyond levels of difference we also observed when comparing different wild-types.

ΔAMP14 flies lacking seven AMP gene families display significantly reduced life span

Drosophila possess many AMPs with possibly redundant or synergistic activities. Thus, AMPs could affect lifespan only when several genes are deleted simultaneously. After evaluating the effect on lifespan of individual AMP genes or genomic clusters of AMPs, we investigated whether combinatory loss of AMPs could impact aging. Using the same approach as described in Hanson et al. [22], we generated four groups of compound AMP mutants that remove different subsets of AMP gene families: *Group A* flies were deleted for *Defensin* and the four *Cecropins*. *Group B* flies were deleted for the structurally related *Drosocin*, *Attacin*, and *Diptericin* families. *Group C* flies were deleted for the two antifungal *Drosomycin* and *Metchnikowin* peptides. And we introduce a new

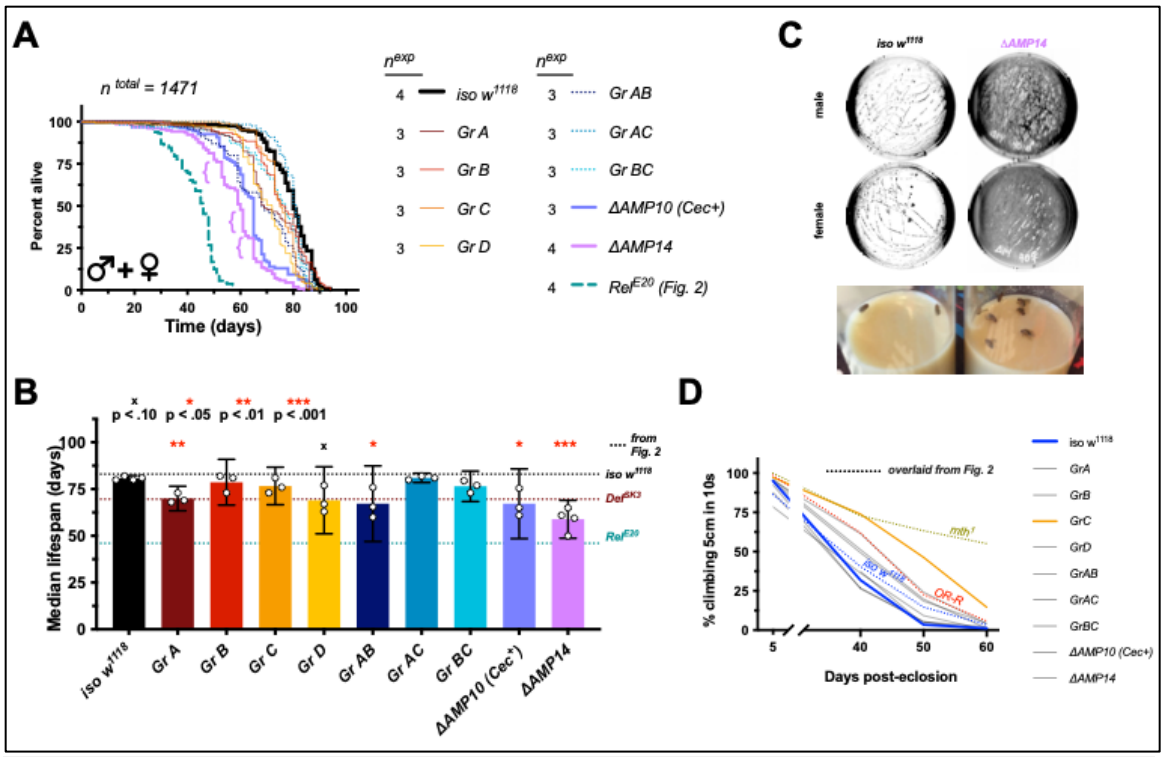

Figure 3: *ΔAMP14* flies have significantly reduced lifespan. A) survival curves of various compound AMP mutants. The lifespan of *Rel*^{E20} from Figure 2 is overlaid for direct comparison. "{" annotations highlight major mortality events in *ΔAMP14* flies. B) Median lifespans of compound AMP mutants. Dotted lines indicate average median lifespans from Figure 2 of *iso w*^{1118} (top), *Def*^{SK3} alone (middle), and *Rel*^{E20} (bottom) for easier comparisons across figures. Statistic summaries (p-value: ˣ, *, **, ***) reflect comparisons to *iso w*^{1118} data specific to Figure 3. C) Top: representative photonegative images of agar plates seeded by the microbiome found in the vial of 40-day old flies. Thick bacterial films in *ΔAMP14* vials are readily visualized by this method, which shows the significantly greater bacterial density (dark parts) compared to iso w^{1118}. Bottom: representative photo of *iso w*^{1118} and *ΔAMP14* food vials revealing discoloured bacterial biofilm alongside a major mortality event ("{" in Fig. 3A). D) Climbing pass rates of AMP group mutants, with climbing curves from genotypes in Figure 2 overlaid for direction comparison. *Group C* is highlighted for having a slightly improved climbing over aging, though this improvement is still minor compared to the climbing competence of *mth*^{1} flies (also see File S4).

isogenic line combining the loss of two recently-described Toll-regulated antifungal peptides: *"Group D"* that are deleted for both *Baramicin A* [13] and the two *Daisho* genes [16]. We screened combined mutants lacking each of these AMP groups, including all the combinations of Groups *A*, *B*, and *C* (i.e. *AB*, *AC*, *BC*, and *ABC* (aka *ΔAMP14*)). We also screened flies lacking 10 AMP genes, but which retain a wild-type *Cecropin* locus (*ΔAMP10*, as used previously [21,22]).

In total we screened nine AMP combinatory genotypes for lifespan and climbing effects (Fig. 3). We found a significant effect of *Group A* mutations on lifespan compared to *iso w*^{1118}, which is not unexpected given this AMP group uses the same 2nd chromosome as the isogenic *Def*^{SK3} mutation as above (Fig. 2), except with the additional loss of *Cecropins* on the third chromosome. Comparisons between the two suggest a non-significant effect of *Cecropin* mutation (*Def*^{SK3} vs. *Group A*, $p > .10$). Deleting other groups of AMPs typically resulted in non-significant effects on median lifespan compared to their *iso w*^{1118} control ($p > .10$ for *Groups B* and *C*, and $p = .092$ for *Group D*). *Group AB* flies also had reduced lifespan compared to *iso w*^{1118}, but were again

comparable to *Def*^{SK3} alone (*Group AB* vs. *Def*^{SK3}, p > .10). *Group AC* and *BC* flies had comparable lifespans to *iso w*^{1118} (p > .10 in both cases), which is notable as *Group AC* flies have the *Def*^{SK3} mutation, but only after an additional round of chromosome II recombination with the isogenic *Mtk*^{R1} chromosome needed to generate the *Group AC* genotype. Deleting ten AMP genes, which included *Defensin*, also had a *Def*^{SK3}-like lifespan (*ΔAMP10* vs. *Def*^{SK3}, p > .10). Thus various combinations of mutants had lifespans comparable to either *iso w*^{1118} or *Def*^{SK3} alone. However, deletion of all 14 classic *Drosophila* AMP genes caused a more pronounced median lifespan reduction than the effect of *Def*^{SK3} alone (*ΔAMP14* vs. *Def*^{SK3}, p = .012), and indeed *ΔAMP14* was also the only genotype with significantly reduced lifespan compared to *OR-R* wild-type from Figure 2 (*ΔAMP14* vs. *OR-R*, p < .001). There was minor variation in climbing competence amongst AMP mutant groups, with *Group C* flies (*Mtk, Drs* double mutants) having slightly improved climbing into old age. However this effect still falls far short of the climbing competence of *mth*^{1} flies into old age (Fig. 3D and see supplementary text).

While deletion of any single AMP gene had little impact on lifespan (barring *Def*^{SK3}), deletion of all 14 classical AMP genes causes lifespan reduction significantly different from wild-type flies. Moreover, recombination of *Def*^{SK3} with another mutation to produce *Group AC* flies yields lifespan comparable to *iso w*^{1118}. This result reinforces the need for careful interpretation of the *Def*^{SK3} mutation effect. Despite this caveat, *ΔAMP14* flies had even further reduced lifespan. The lifespan of *ΔAMP14* flies is intermediate between *iso w*^{1118} and *Rel*^{E20}, indicating *Relish* likely impacts fly lifespan through processes other than AMP regulation.

ΔAMP14 flies suffer microbial dysbiosis with aging

During our studies, we noticed that the food surface of the vials containing *ΔAMP14* flies became discolored and sticky upon aging, suggesting microbial proliferation (Fig. 3C bottom). The *Drosophila* microbiome is found both in the gut and in the external environment due to constant ingestion and fecal deposition [68–70]. We suspected this change in the fly food appearance in *ΔAMP14* upon aging could be linked to a change in the microbiome, as we have recently described a role for AMPs in regulating *Acetobacter* using *ΔAMP14* flies [25]. Sticky and discolored food was also observed in *Rel*^{E20} flies, which also do not express antimicrobial peptides, and indeed both *ΔAMP14* and *Rel*^{E20} flies suffer increased dysbiosis over aging [25]. To test if microbiome load was associated with early mortality of *ΔAMP14*, we monitored bacterial abundance on the food medium. To do this, we emptied vials where flies had been present for two days, added glass beads to those vials, shook vials with beads for 10 seconds, then placed those beads on agar plates and spread vial microbes by rolling the beads over the agar for 10 seconds. By 40 days post-eclosion (dpe), plating of vial contents using beads confirmed far higher microbe loads in aged *ΔAMP14* vials compared to *iso w*^{1118} (Fig. 3C top,). Moreover, in the time period between 50-70dpe, most major mortality events in *ΔAMP14* vials were associated with sticky and discolored bacterial food (example photo in Fig. 3C bottom). These major mortality events are also seen as precipitous drops in Fig. 3A (indicated with "{") survival curves in *ΔAMP14* flies, and similar trends were observed for *ΔAMP10*.

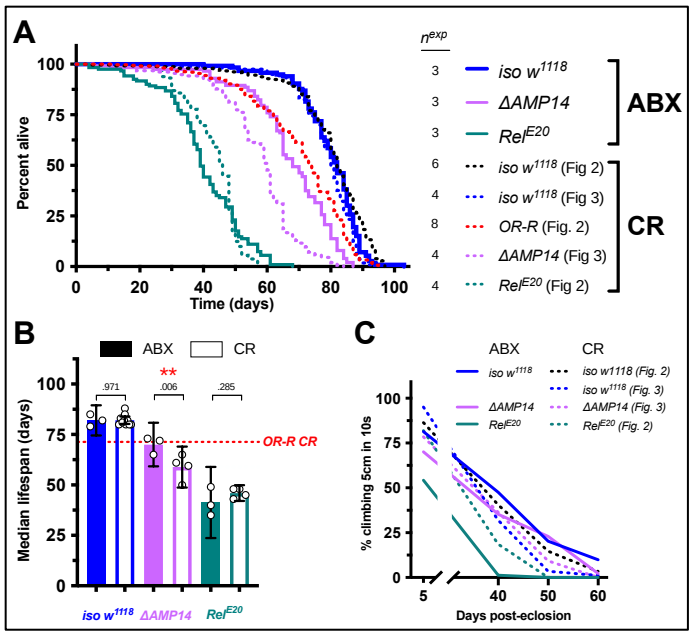

Figure 4: Microbiome depletion rescues the *ΔAMP14* fly lifespan to wild-type levels. A) Survival curves, including both antibiotic-reared flies (ABX), and also conventionally-reared (CR) lifespans from previous figures as dotted lines for direct comparison. B) Median lifespans, including both ABX and CR fly lifespans for direct comparison (conventionally-reared *iso w*^{1118} and *Rel*^{E20} lifespans from Fig. 2C). The dotted line indicates *OR-R*-CR median lifespan from Fig. 2, which is similar to the lifespan of *ΔAMP14*-ABX flies. C) Climbing pass rates of ABX (solid lines) and CR (dotted lines) flies at 5, 40, 50, and 60 days post-eclosion.

We conclude that *ΔAMP14* flies thus suffered increased microbe load within vials with aging, agreeing with an increase in gut microbiome abundance with aging shown previously using gnotobiotic flies [25]. This suggests that precocious aging observed in *ΔAMP14* flies could be indirectly caused by the impact of AMPs on the microbiome.

Lifespan of *ΔAMP14* flies can be rescued significantly by rearing on antibiotic medium

To test whether the effect of AMP mutation on lifespan was linked to changes in the microbiome, we performed the same lifespan experiments in germ-free conditions using *iso w*^{1118}, *ΔAMP14*, and *Rel*^{E20}. For this, we first bleached embryos, and then kept larvae and emerging adults on antibiotic food media for their entire lifespan. In our hands, antibiotic-reared *iso w*^{1118} flies have a similar lifespan to conventionally reared flies. Likewise, in our hands *Rel*^{E20} mutants had similar lifespan whether reared conventionally or in antibiotic conditions. However, rearing *ΔAMP14* flies on antibiotics significantly rescued their lifespan (Fig. 4A-B, *ΔAMP14* ABX vs. CR, $p = .006$), even up to *OR-R* wild-type levels (*ΔAMP14-ABX vs. OR-R*-(Fig. 2), median lifespan $p > .10$). Climbing competence was largely unaffected by rearing in germ-free conditions (Fig. 4C).

These results suggest that the short lifespan of *ΔAMP14* flies primarily relies on the impact that antimicrobial peptides have on the microbiome through aging. The observation that antibiotics treatment rescues *ΔAMP14* but not *Relish* mutant lifespan suggests the short lifespan of *Rel*^{E20} flies depends on effects other than the microbiome and independent of AMPs.

Discussion

A number of studies done in *Drosophila* and *C. elegans*, but also vertebrates, have implicated AMPs in processes as diverse as behavior, neurodegeneration, tumor clearance, and aging. In mammals, some AMPs function as pro-inflammatory cytokines, and as such could influence these processes by disrupting homeostasis when chronically expressed [10,12,42]. However, to date there is no evidence of a cytokine role for AMPs in *Drosophila* [8]. AMPs can also disrupt the membranes of aberrant host cells, that may become more negatively charged due to the exposure of phospholipids such as phosphatidylserine, a well-known "eat-me" signal that promotes the phagocytosis of apoptotic cells [71–73]. Two studies have now implicated AMPs in the control of tumors in vivo in *Drosophila* [26,27], providing a proof of principle that AMP action can target self-tissue. Thus, AMPs could have a role in tissue maintenance with consequences on the aging process. Moreover, AMPs have a number of properties in common with neuropeptides, often being cationic and amphipathic [12,74], alongside descriptions of AMP-like genes undergoing downregulation in the brain after exposure to pheromones [75], modulating sleep [76,77], memory [78], and affecting behaviors [13,79,80]. Thus, it cannot be excluded that AMPs modulate neuronal activity, a process that could impact lifespan.

There is an increase of AMP gene expression observed upon aging, though it was unclear if AMPs directly affect lifespan, or if this activation is just a secondary consequence of aging resulting from, for instance, dysbiosis and gut barrier dysfunction [25,44–46]. Using flies carrying null mutations in AMP genes, we find no evidence that individual AMPs are so essential to host physiology that they have a notable impact on fly lifespan. Overall, the lifespan of flies mutated for individual genes or clusters of AMPs, *Bomanins*, or *Daishos*, did not differ from the wild-type. The climbing activity of these mutants were also similar to wild-type flies. The only possible exception to this was the apparent lower lifespan of *Def* mutants. Given a role for Defensin in tumor clearance [26], it is possible that Defensin is required to eliminate senescent or aberrant cells over aging. Nevertheless, we cannot exclude that the somewhat shortened lifespan of *Def* mutant flies was not linked to *Def* mutation itself, but due to the presence of one or several cryptic mutations that were not removed during the isogenization process due to their proximity to the *Def* locus. Accordingly, silencing *Defensin* with RNAi did not reduce lifespan, and flies with certain combinations of Def^{SK3} and other AMP mutations were long-lived (e.g. *Group AC* flies in Fig. 3). Alternately, complex interactions amongst AMPs could provide a protective effect against *Defensin* mutation, as AMP interactions can synergize to prevent damage to host membranes [81]. Overall, our study suggests that individual AMPs do not affect lifespan beyond levels of difference we also saw when comparing wild-types and/or classic aging mutant flies (e.g. mth^{1}), a result that contradicts other studies using RNAi or overexpression methods. However, our results do support a role for AMPs in regulating the microbiome over aging, which likely has indirect effects on lifespan.

We cannot exclude that additional AMP mutant combinations could reveal a stronger impact of AMPs on lifespan. Indeed, a number of immune-induced peptides that

could be AMPs await description [8,82], and additional deletion of those peptides could explain the lifespan difference between *ΔAMP14* and *Relish* mutants. Use of other rearing conditions could also reveal a role for AMPs not found here. As part of this study, we also monitored AMP expression upon aging in four wild-type backgrounds (a lab standard w^{1118}, *Exelexis*, *OR-R*, and *Canton-S*) by separating out dissected heads and bodies (thorax + abdomen). In our hands, we observed increased expression of AMP genes in fly bodies aged 40 days compared to young flies, though the extent differed by genotype (Table S1 and File S2). However, we saw no marked AMP increase in the head with aging, including in a separate experiment following flies over multiple time points (File S2). Indeed, when AMPs were upregulated in the head upon aging, this was often coupled with a stronger upregulation in the body, suggesting that this increase of AMP expression in the head could derive from head-specific fat body responding like the fat body in the thorax and abdomen. We also tested if glia-specific *Relish* knockdown (via *Repo-Gal4* and *UAS-Rel-IR*) could rescue lifespan, as many studies suggest a role of *Relish* in neurons/glia to rescue neurodegenerative syndromes [29–32], but if anything we saw the opposite effect: *Repo>Rel-IR* flies had shorter lifespan than genetic controls, and we found that *Repo-Gal4* alone had improved climbing into old age, suggesting a genetic background effect on aging unrelated to RNAi using this driver (Fig. S4). While *Repo>Rel-IR* can rescue various models of neurodegeneration, taken together our results suggest AMPs are not especially upregulated in the head upon aging (except when also upregulated in the body), and that glia-expressed *Relish* does not have a major deleterious role in lifespan using our standard rearing conditions. Differences between research groups (local microbiome composition, food recipe, etc…) could account for these conflicting results, and should be considered when comparing our AMP mutant results to the larger field.

Importantly, our study suggests that AMPs collectively affect fly lifespan through their impact on the microbiome. Several studies have investigated the role of the gut microbiome on lifespan with mixed results [56–58]. It could be that discrepancies between studies reporting an impact of the microbiome on aging results from a complex interaction between nutrition and gut bacteria. In poor or unbalanced diets, the microbiome could have a positive impact on lifespan by extracting more nutrients from the food . In contrast, on a lab standard nutrient-rich diet, as we have used in this study, the microbiome could have less impact. Consistent with this, we did not see major differences between axenic and conventionally raised wild-type flies in this study. Previous studies have already revealed the role of Imd signalling in controlling microbiome load and diversity, preventing dysbiosis [46,88–90]. More recently, use of *ΔAMP14* flies revealed a key role of AMPs to control bacteria in the gut, notably the Gram-negative bacterium *Acetobacter*, and accordingly, *ΔAMP14* flies have increased microbiome load upon aging ([25] and Fig. 3C). Our present study shows that the action of AMPs preserves lifespan, and that this effect is largely due to their impact on the microbiome. Indeed, *ΔAMP14* flies raised axenically have lifespan in the range of wild-type flies. Thus, the impact of AMPs on lifespan is consistent with their well-established microbicidal activity. This finding also agrees with studies showing that microbiome dysregulation negatively impacts lifespan [88,89]. Interestingly, raising *Relish* mutant flies in axenic conditions did not lead to lifespan extension, indicating that *Relish*, and

likely the Imd pathway, have a much more profound impact on host physiology independent of AMP regulation. In line with this, *Relish* and the Imd pathway have been implicated in cell competition [91,92], metabolism [93,94], and enterocyte delamination [46,95], processes that likely impact lifespan. It is interesting to note that the food media of *ΔAMP14* and Rel^{E20} flies was enriched in bacteria, agreeing with elevated bacterial loads in these flies with aging shown previously [25]. External microbes colonize the *Drosophila* gut, and gut microbes are released into the external environment as part of excreta [68,70,86,96]. Thus, it was expected that an increased gut microbiome load in the absence of AMPs would result in high bacterial load in the fly food medium. Although we reared flies in a vial in artificial lab conditions here, it is tempting to speculate that AMPs expressed in the gut could not only shape the gut microbiome, but also environmental bacteria. The increased load in the food medium could therefore rely either on AMP-mediated control of the gut microbiome, or on external AMPs secreted into the food medium. This would suggest that AMP mutation can exacerbate microbiome effects from both within the fly and in the external vial environment.

During our study, we experienced a number of challenges to lifespan data interpretation. Notably, our reference wild-type was previously infected with *Drosophila nora virus*, which had a greater deleterious effect on lifespan in our hands compared to a previous study (see Fig. 1A and Habayeb et al. [64]). Thus, we interpreted our early results as if AMP deletion extended lifespan to a great extent compared to their isogenic wild-type controls. However, our ultimate findings, including standard genotypes and different conditions, instead highlight that loss of AMPs does not extend lifespan; if anything, they show the opposite. We believe that infection by *nora virus*, which we found infecting many of our fly stocks, represents a serious threat to lifespan studies and is an essential consideration for future fly longevity research.

In conclusion, our study reveals a key role of AMPs in the aging process: but only indirectly, through their effect on the microbiome. We cannot exclude that certain contexts could reveal an impact of AMPs on aging, such as conditions found in individuals that have aging-associated diseases like cancer or precocious neurodegeneration, uncommon in standard wild-types. However, here we do not find evidence of AMPs directly impacting aging in a striking way. We are still far from understanding the complex relationship between the immune system, senescence, and aging, which requires further investigation.

Materials and Methods

Drosophila rearing conditions

Drosophila stocks used in this study, including genotype descriptions, are listed in File S3. Food media used the following recipe (per 600mL): 3.72g agar, 35.28g cornmeal, 35.28g yeast extract, 36mL grape juice, 2.9mL propionic acid, and 15.9mL Moldex. Antibiotic media also contained final concentrations of 50ug/mL Ampicillin, 50ug/mL Kanamycin, 10ug/mL Tetracycline, and 10ug/mL Erythromycin. Flies were flipped three times per week (Monday, Wednesday, Friday), and vials were left on their side to ameliorate the effect of the food medium stickiness on mortality with aging, by allowing flies falling to the ground to drop onto plastic rather than the food surface. This precaution was taken as vial conditions differ markedly in specific immune-deficient genotypes [25].

To clear flies of *nora virus*, embryos were collected from grape juice agar plates, rinsed with distilled water, and left to soak in 3% bleach for 3 minutes. Embryos were then rinsed twice in distilled water for one minute each. This protocol was also used to clear the microbiome of antibiotic-reared flies, whereafter we placed embryos directly on antibiotic medium for germ-free experiments.

Lifespan experiments

Lifespan experiments were conducted from 2017 to 2022. Flies were allowed to emerge and mate randomly for ~3 days prior to separating males and females. Then, groups of 20 males or 20 females (mated) were flipped three times per week (Monday, Wednesday, Friday) to measure fly lifespan in 90x15mm polystyrene vials.

We used a Cox proportional hazard (CoxPH) mixed effects model to initially analyze lifespan effects, with experimental replicate and biological sex as interaction terms in R version 3.6.3. In both cases, experimental replicate and sex were significant contributors to the model ($p < .001$). We report our final CoxPH model statistics within the figures for data transparency, including the Hazard Ratio (HR) and p-value. Our impression from the initial data analysis was that the CoxPH model was overly sensitive to minor variation around the geometric mean lifespan of *iso* w^{1118} control flies, exacerbated by the large sample sizes used and the many comparisons performed in our study inflating the likelihood of Type I (false positive) statistical errors. Even if minor differences in lifespan were genuine, their ultimate importance was questionable when compared to other genetic backgrounds (e.g. *OR-R*, mth^{1}), particularly given variable mutation types and transgene insertions used for different AMP mutations (i.e. point mutation, genomic deficiency, $white^{+}$, 3xP3-EGFP, or 3xP3-dsRed). We thus preferred to focus on each experiment as if that population of flies represented a single sampling. Therefore we treated our data as if we had e.g. n = 3 per genotype (3 experiments), rather than n = 120 (60 males and 60 females) per genotype across 3 experiments. For this purpose, we decided to use median lifespans as our primary readout. Median lifespans were analyzed

using one-way ANOVA with Holm-Sidak's multiple test correction implemented in Prism v9.3.1, or one-way ANOVA with Tukey's HSD correction in R 3.6.3.

Climbing pass rates

We paired our lifespan data with the gravotaxic locomotor climbing assay to provide an independent metric of aging [97]. This assay assesses the general locomotor competence of the fly, which is often used as a readout of neurodegeneration, but can also reflect generic aging effects (e.g. muscle weakness). Climbing pass rates were filmed for flies at 5, 40, 50, and 60 days post-eclosion ±2 days, and analyzed later manually. A pass was considered if a fly climbed 5cm within 10s of being tapped to the bottom of the vial. Flies were transferred to a chamber made of two empty vials stacked atop each other to provide ample room to climb upwards without reaching the ceiling or disrupting other flies. Two sets of broken markings were made with permanent marker at 2.5cm and 5cm on the lower vial as reference points, though we ultimately report only 5cm climbing rates given similar trends between the two. At each time point, an initial tapping down was performed to associate the flies with their new environment and encourage climbing behaviour, as we found the first repetition of this experiment often had fewer climbers than subsequent repetitions. After this initial association, three technical repeats of the climbing assay were performed, and final values represent the average of these three technical repeats. All climbing measurements were taken between 2pm-5pm to ensure a consistent measurement timeframe.

This assay can be a measure of both climbing speed and/or readiness of response: for instance, older flies sometimes suffered temporary seizures after being tapped down, or might climb slower, more erratically, or simply to a lesser extent than younger counterparts. In most cases, climbing pass data mirrored lifespan data in terms of relative trends. We thus have no suspicion that AMP mutations, individually or collectively, greatly affect locomotory competence with aging. The only noteworthy exception to mirrored trends between lifespan and climbing was *methuselah* mutant flies, which had wild-type lifespans, but improved climbing competence into older age at both 25°C and 29°C.

Microbiome monitoring

We monitored the microbiome of compound AMP mutants and antibiotic-reared flies by checking plated vial food contents on MRS + Mannitol agar, a medium amenable to both Lactobacilli and *Acetobacter*. Specifically, at both ~20 and ~40 days we took vials where flies had been present for two days, added 5 glass beads to the vials, shook the glass beads in vials for 10s, then transferred glass beads to MRS+Mannitol agar plates and shook plates for 10s, then removed the beads and left plated microbes to grow overnight at 29°C. Vial microbiome loads were checked on Wednesdays. We chose Wednesdays rather than a precise age (e.g. exactly 20dpe) to ensure that flies entered the plating time point after experiencing a similar regimen of flipping; i.e. in our experiments, we only plated microbes from vials where flies had spent the past five days with ~3 days in a vial accruing microbes over the weekend (Fri-Mon), and the next two days depositing

microbes in the vial that was ultimately measured (Mon-Wed). This design was chosen based on demonstrations that flipping regimen drastically affects microbiome load [69,98,99]. Beyond ~40 days, comparisons were not equal due to onset of mortality in AMP mutants and associated drops in vial fly density.

In antibiotic experiments, microbes were never detected from overnight growth using this method to monitor vial microbiome loads.

Gene expression assays

Gene expression was performed using primers listed in File S3 with PowerUP SYBR Green Master Mix, using the PFAFFL method of qPCR quantification with Rp49 as the reference gene [100]. RNA was extracted using TRIzol according to manufacturer's protocol. cDNA was reverse transcribed using Takara Reverse Transcriptase.

Dissections of heads from bodies was performed in ice cold PBS, and tubes containing pools of 20 heads or bodies were kept at -20°C until after TRIzol was added to prevent RNA degradation before sample processing.

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Figure captions

Figure 1: *Drosophila nora virus* significantly reduces lifespan of DrosDel *iso* w^{1118} flies. A) Comparison of lifespan from early experiments using two wild-types (*iso* w^{1118} and *OR-R*) alongside compound AMP mutants lacking *Def*, *Dro*, *AttA,B,C*, *Mtk*, and *DptA,B*, which are deleted in *ΔAMP Chr2* flies. B) Effect of *nora* clearance on *iso* w^{1118} lifespan. Number of independent experiments (n^{exp}) and total flies (*N*) is reported. C) *Nora* titres measured by CT value in 18°C source stocks (Stocks) or *nora*-positive flies aged 3+ weeks kept at densities of 20 flies per vial (Aged flies). CT values represent *nora* titre from 5ng total fly RNA per 10µL qPCR reaction.

Figure 2: Individual AMP gene deletions do not drastically affect lifespan. A) Cumulative lifespans of male and female flies with various genetic backgrounds. B) Cumulative lifespans of single gene AMP mutants. Most AMP mutant lifespans (black lines) cluster around the wild-type (blue line), except Def^{SK3}. $Bom^{\Delta 55C}$ is also noted as an outlier perhaps living slightly longer than *iso* w^{1118}. C) Median lifespans where each data point represents one replicate experiment (cumulative of 20 males and 20 females). Median lifespans analysis suggests that the only AMP mutation significantly differing from *iso* w^{1118} was Def^{SK3}. Of note, the impact of *Def* on lifespan was not corroborated using *Defensin* RNAi (Fig. S3). $Bom^{\Delta 55C}$ median lifespans were not different from *iso* w^{1118}. Horizontal dotted lines indicate median lifespans of *iso* w^{1118} (top), *OR-R* (middle), or Rel^{E20} (bottom). Statistic summaries (p-values: ˣ, *, **, ***) reflect comparisons to *iso* w^{1118}. D) Climbing pass rates suggest most AMP mutants climb like wild-type flies, while *methuselah* mutants uniquely retain climbing competence into old age (see Fig. S3 for detailed analysis).

Figure 3: *ΔAMP14* flies have significantly reduced lifespan. A) survival curves of various compound AMP mutants. The lifespan of Rel^{E20} from Figure 2 is overlaid for direct comparison. "{" annotations highlight major mortality events in *ΔAMP14* flies. B) Median lifespans of compound AMP mutants. Dotted lines indicate average median lifespans from Figure 2 of *iso* w^{1118} (top), Def^{SK3} alone (middle), and Rel^{E20} (bottom) for easier comparisons across figures. Statistic summaries (p-value: ˣ, *, **, ***) reflect comparisons to *iso* w^{1118} data specific to Figure 3. C) Top: representative photonegative images of agar plates seeded by the microbiome found in the vial of 40-day old flies. Thick bacterial films in *ΔAMP14* vials are readily visualized by this method, which shows the significantly greater bacterial density (dark parts) compared to *iso* w^{1118}. Bottom: representative photo of *iso* w^{1118} and *ΔAMP14* food vials revealing discoloured bacterial biofilm alongside a major mortality event ("{" in Fig. 3A). D) Climbing pass rates of AMP group mutants, with climbing curves from genotypes in Figure 2 overlaid for direction comparison. Group C is highlighted for having a slightly improved climbing over aging, though this improvement is still minor compared to the climbing competence of mth^1 flies (also see File S4).

Figure 4: Microbiome depletion rescues the *ΔAMP14* fly lifespan to wild-type levels. A) Survival curves, including both antibiotic-reared flies (ABX), and also conventionally-reared (CR) lifespans from previous figures as dotted lines for direct

comparison. B) Median lifespans, including both ABX and CR fly lifespans for direct comparison (conventionally-reared *iso* w^{1118} and Rel^{E20} lifespans from Fig. 2C). The dotted line indicates *OR-R*-CR median lifespan from Fig. 2, which is similar to the lifespan of *ΔAMP14-ABX* flies. C) Climbing pass rates of ABX (solid lines) and CR (dotted lines) flies at 5, 40, 50, and 60 days post-eclosion.

Supplementary figures, tables, and files

Figure S1: Lifespan of various fly genotypes at 29°C. A) *Relish* mutant (Rel^{E20}) and compound AMP mutants (*ΔAMP14*) suffer significantly reduced lifespan at 29°C. B) Median lifespan data from Fig. S1A. C) Climbing pass rates of flies reared at 29°C. mth^{1} flies are the only fly line that retains climbing competence into old age.

Figure S2: AMP gene silencing ubiquitously or in glia emphasizes the importance of reference genotypes for lifespan context. A) Comparison of multiple *Actin5C-Gal4>AMP-IR* lines shows possible major lifespan effects of three specific *Gal4>AMP-IR* combinations: *Act>DptA-IR*, *Act>Mtk-IR*, and *Act>Drs-IR* but not *Act>DptB-IR* or *Act>Def-IR*. B) However, these lifespan differences are not especially striking in the context of additional genetic background controls and references. For instance, *Act>Drs-IR* lifespan is not very different from *OR/Drs-IR* control or *Repo>Drs-IR*. Similarly, despite a seeming lifespan extension effect by *Act>DptA-IR*, we found no lifespan effect in *Dpt* mutants (Fig. 2).

Figure S3: Climbing pass rate data from individual mutants. A) Climbing pass rate curves over aging in males and females, with *Drs* and *Mtk* additionally highlighted for having slightly improved climbing competence at older ages. AMP mutants displaying climbing rate within wild-type range are left with solid black lines (left panels). Of note, Drs^{R1} and Mtk^{R1} are the only mutations which are caused by the insertion of $white^{+}$ transgenes. Thus, we cannot exclude that their improved climbing effect results from the presence of $white^{+}$ compared to *iso* w^{1118} and other AMP mutants. In line with this idea, *OR-R* $white^{+/+}$ wild-type flies similarly trended towards better climbing competence than *iso* w^{1118} flies. B) Climbing pass rate of select genotypes broken down by individual vial replicates per time point. Black horizontal bars show the mean, and error bars represent standard deviation. Statistical comparisons to *methuselah* mutants are highlighted as a reference with prolonged climbing competence into old age. ns = not significant, $p < .05$ *, $p < .01$ **.

Figure S4: Silencing *Relish* in glia does not extend lifespan. A) Silencing Relish in glia provides no lifespan benefit (*Repo>Rel-IR* genotype: *UAS-Rel-IR/+; Repo-Gal4/+*). B) Median lifespans of *Repo* and *Rel-IR* combinations. Differences between *Repo>Rel-IR* and respective controls were not significant. C) Climbing pass rates of flies with various combinations of *Repo* and *Rel-IR* systematic crosses. Left panel shows linear progression of climbing competence loss, right panel shows individual data points. The *Repo-Gal4* genetic background seems to improve climbing competence into old age independent of

the RNAi construct. Of note, the lifespan *OR-R* wild-type flies in these experiments was poor compared to the eight previous *OR-R* experiments shown in Fig. 2. We include the Fig. 2 *OR-R* lifespan and climbing (and mth^1 climbing) in the present figure (A, C) for context, and for ease of direct comparisons.

Table S1: No consistent signal of high AMP expression in the head upon aging. We aged four wild-types (*Oregon-R, Exelexis,* w^{1118} *VDRC, and Canton S*) for 40 days and compared AMP expression in 40 day old flies to 5 day old flies, specifically in heads or decapitated bodies. While Imd-responsive AMPs were somewhat consistently upregulated in the body with aging, there was little consistent upregulation of AMPs in the head with aging. When present, upregulation was often minor compared to the expression seen during a systemic immune response for those AMP genes (e.g. at 40dpe *DptA* was induced anywhere from 2-50x in the head, or 5-130x in the body, while induction upon infection often reaches 500-1000x using the same normalization procedure). Underlying data and additional qPCR data from a separate experiment following AMP expression in the heads of aging flies at 8, 15, and 30 days are provided in File S2.

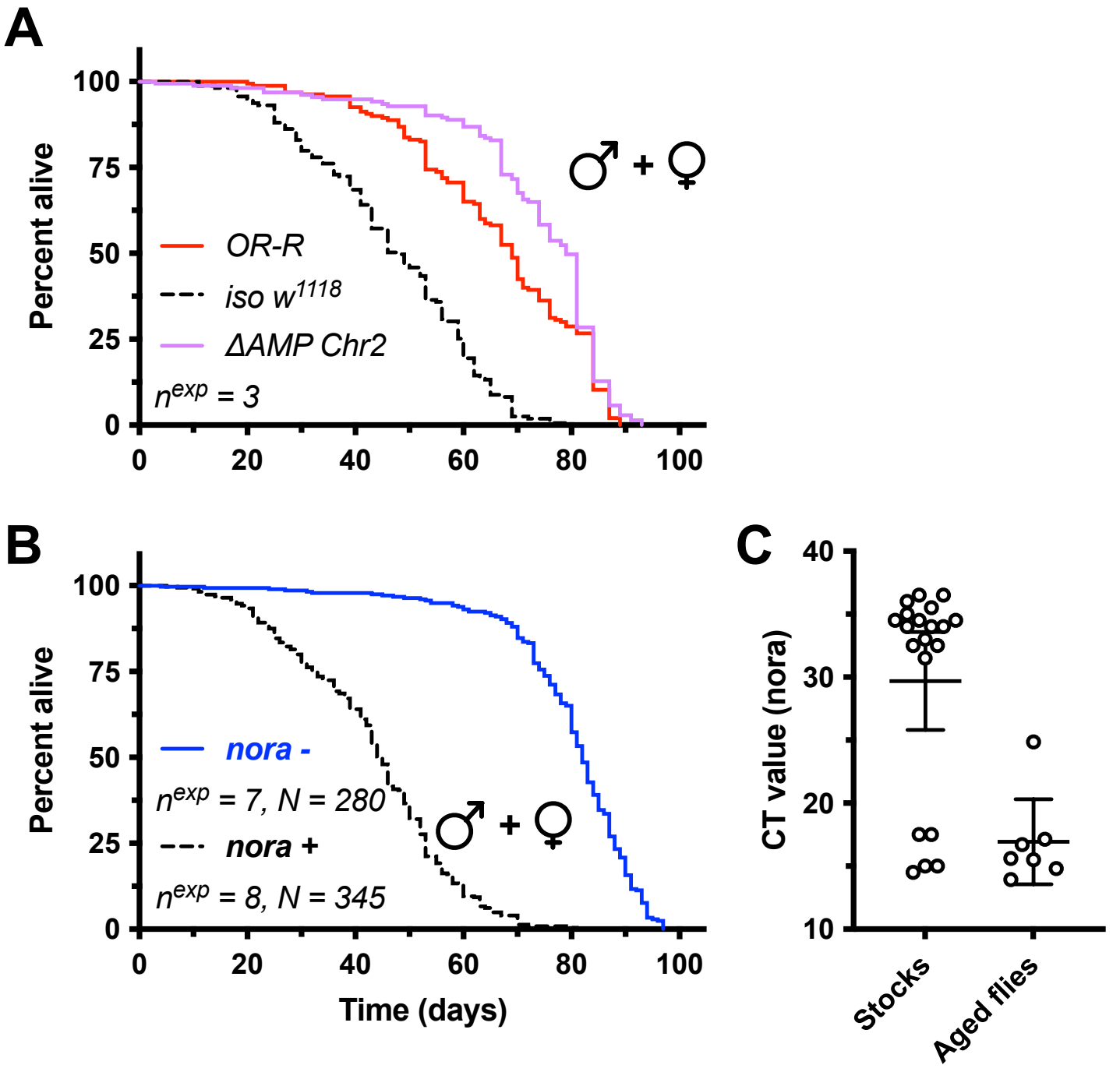
File S1: Median lifespan and other summary statistics from all genotypes across all experiments, separated by genotype * experiment * sex. Tabs within this excel file are named according to which experiment they relate to. Full survival data are uploaded in a separate file.

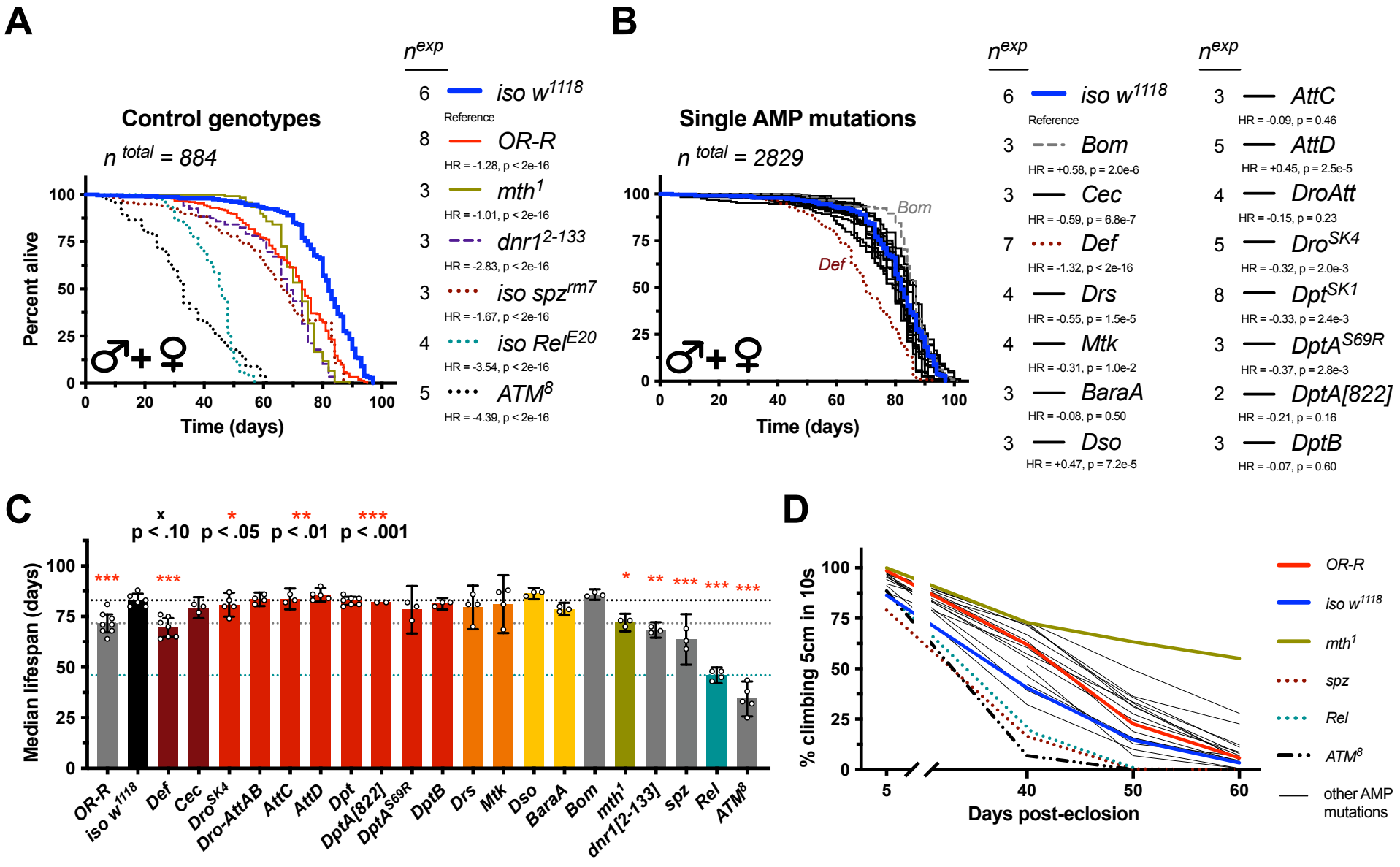
File S2: AMP expression data in the head. Contains data related to Table S1 for four wild-type flies collected at 5dpe and 40dpe. Includes additional data on AMP expression in the heads of aged flies at 8, 15, and 30 days post-eclosion.

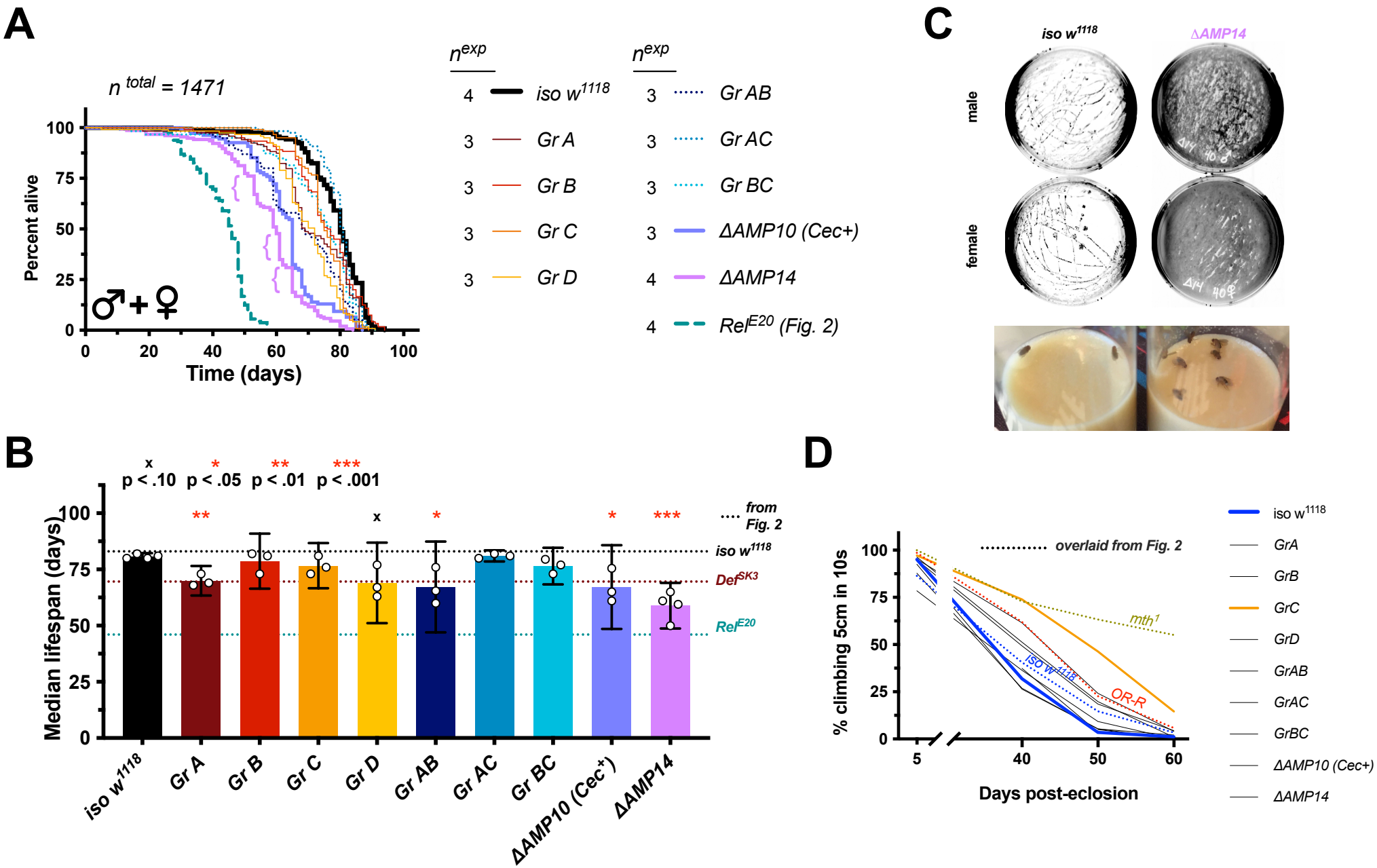
File S3: Description of fly stocks and qPCR primers used in this study.

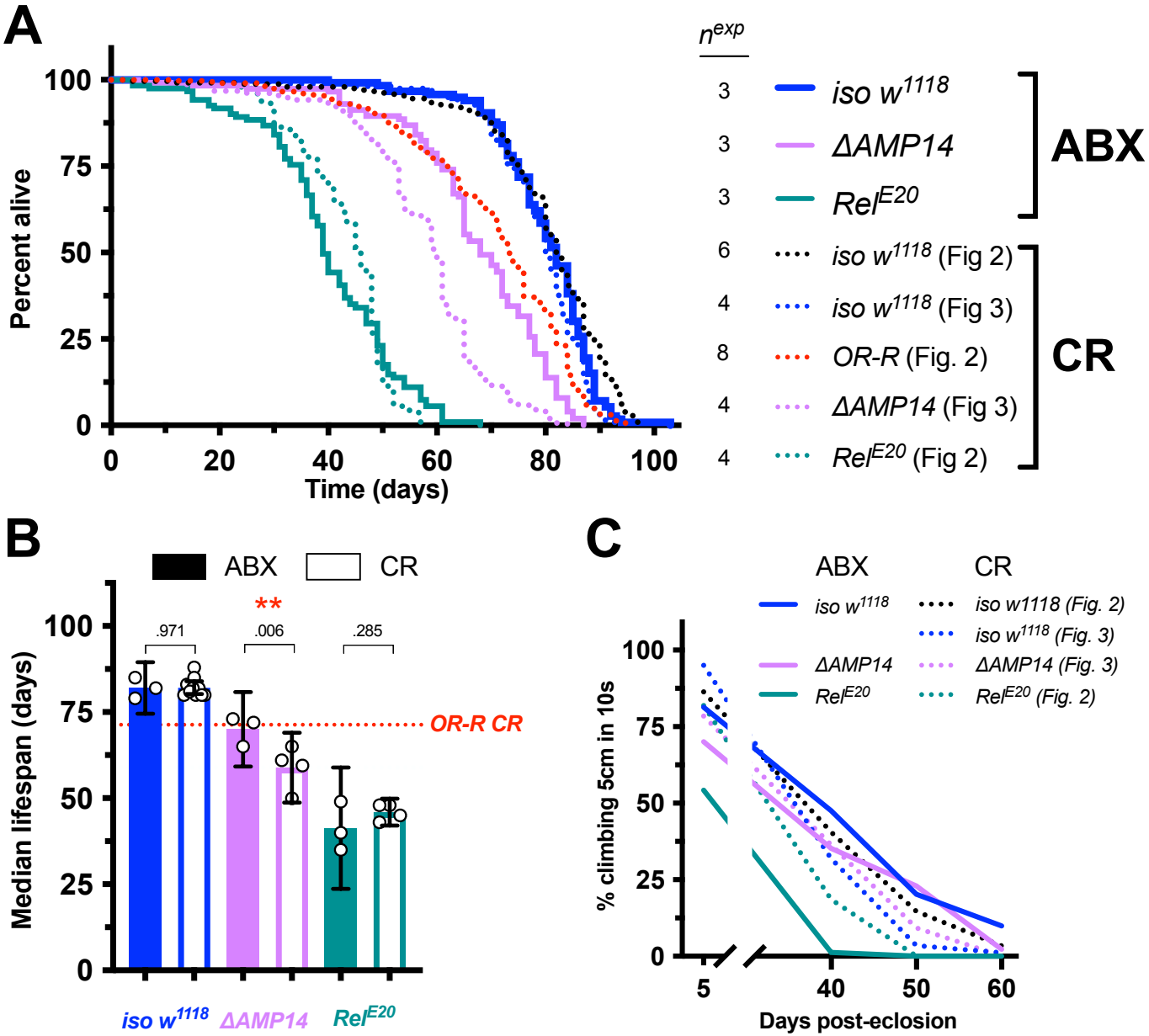
File S4: Supplemental text. This supplementary discussion addresses issues and limitation of our study that we encountered when combining mutations in the DrosDel isogenic background. We discuss the use of median lifespan as our primary readout. There is also discussion of the unique, but minor, climbing effect of *Mtk* and *Drs* mutation in the light of a recent study, but also with the caveat of Mtk^{R1} and Drs^{R1} having a $white^+$ transgene insertion that may affect aging and neurodegeneration.

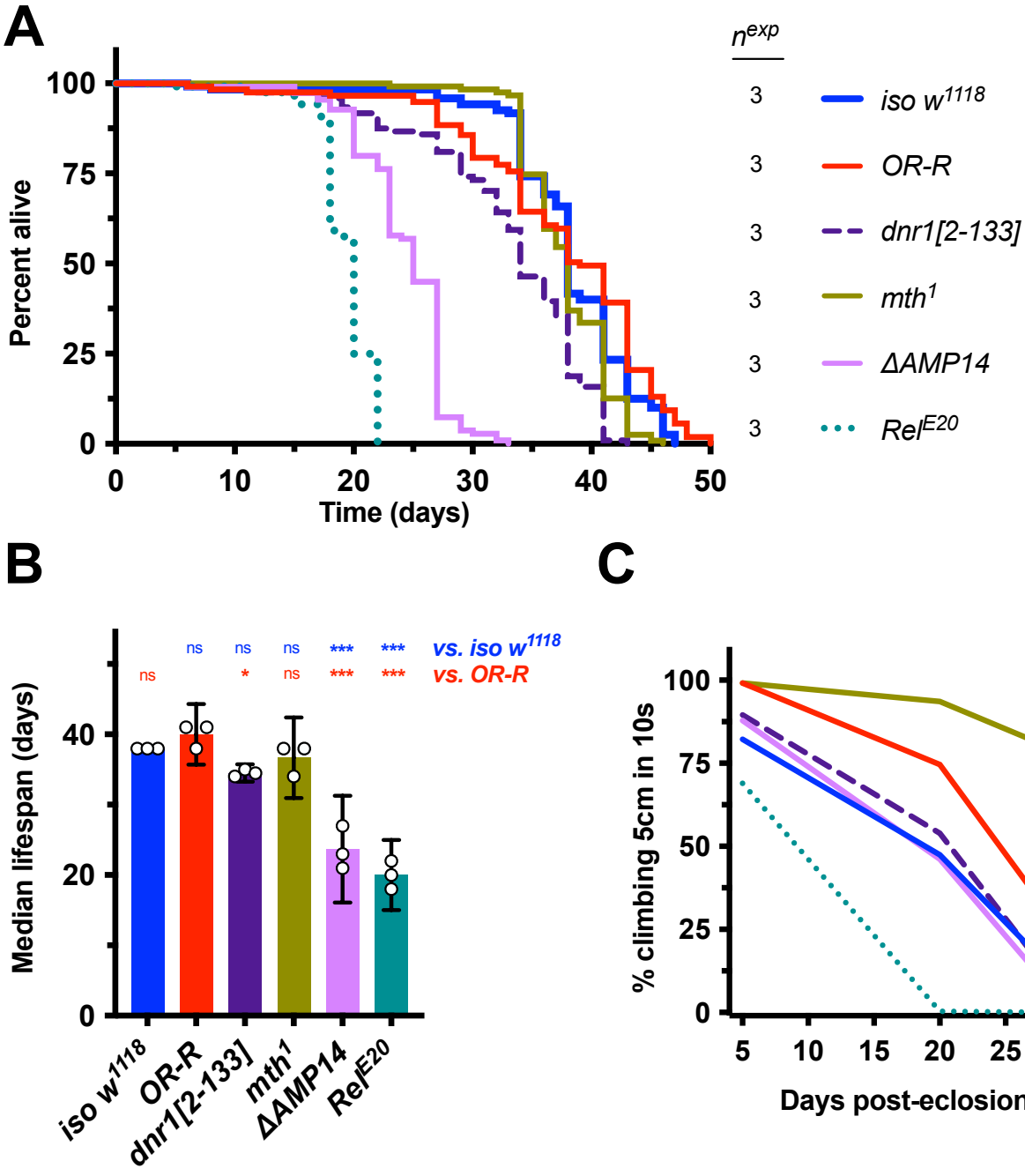
File S5: Raw data for all experiments.

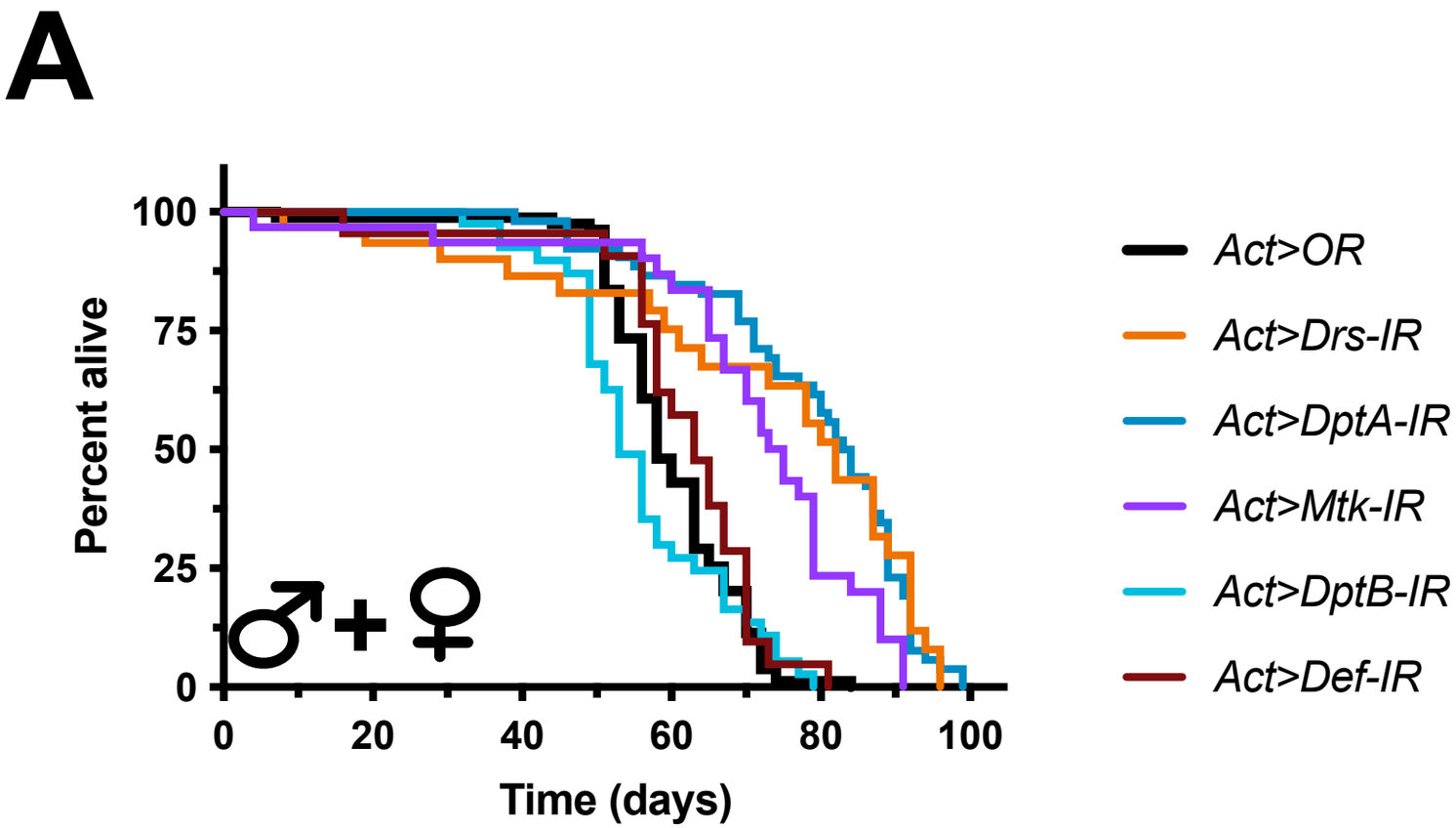

A
Percent alive
100
75
50
25
0
0
20
40
60
80
100
OR-R
iso w1118
ΔAMP Chr2
nexp = 3
♂ + ♀
B
Percent alive
nora -
nexp = 7, N = 280
nora +
nexp = 8, N = 345
♂ + ♀
Time (days)
C
CT value (nora)
40
30
20
10
Stocks
Aged flies

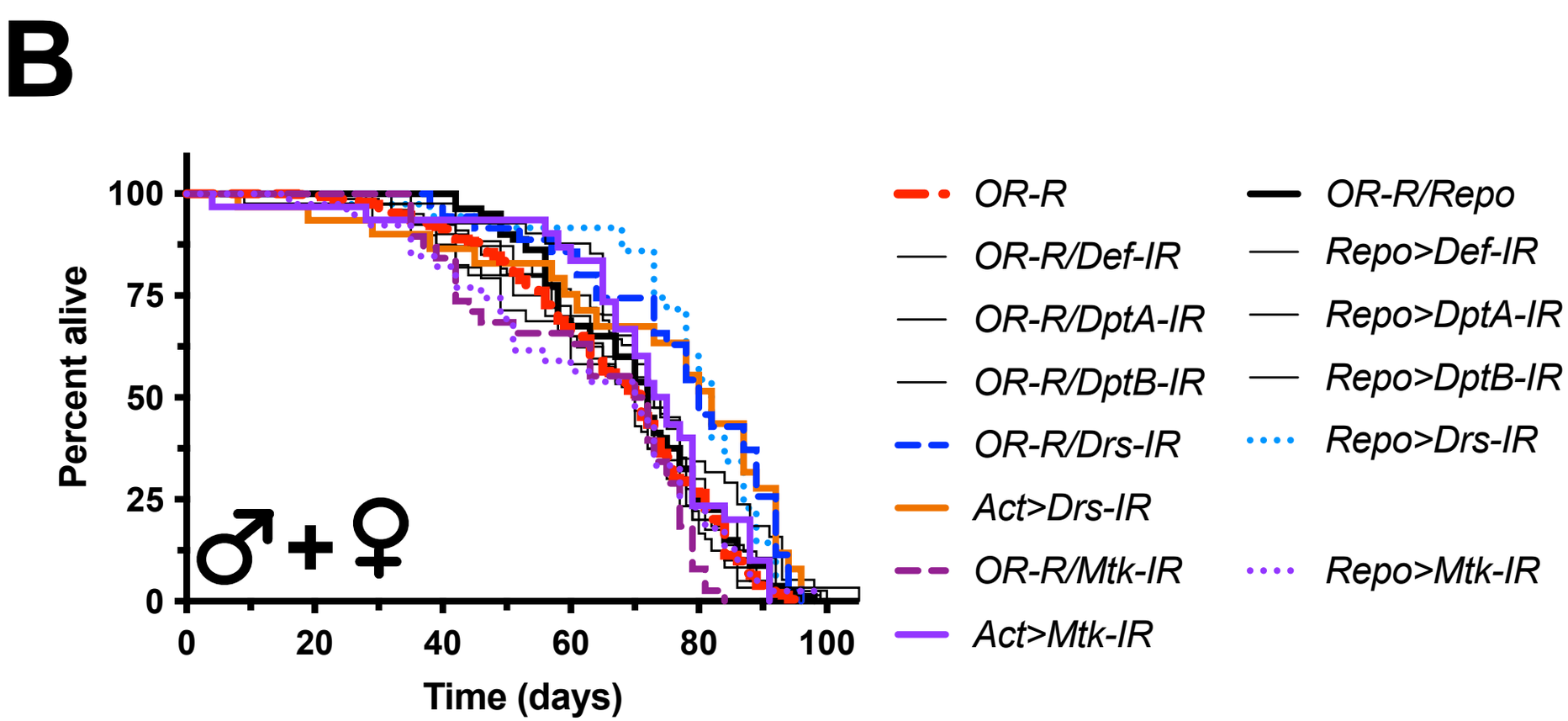

A
Control genotypes
n total = 884
Percent alive
Time (days)
♂+♀
n exp
6 iso w1118
Reference
8 OR-R
HR = -1.28, p < 2e-16
3 mth1
HR = -1.01, p < 2e-16
3 dnr12-133
HR = -2.83, p < 2e-16
3 iso spzrm7
HR = -1.67, p < 2e-16
4 iso RelE20
HR = -3.54, p < 2e-16
5 ATM8
HR = -4.39, p < 2e-16
B
Single AMP mutations
n total = 2829
Bom
Def
Time (days)
♂+♀
n exp
6 iso w1118
Reference
3 Bom
HR = +0.58, p = 2.0e-6
3 Cec
HR = -0.59, p = 6.8e-7
7 Def
HR = -1.32, p < 2e-16
4 Drs
HR = -0.55, p = 1.5e-5
4 Mtk
HR = -0.31, p = 1.0e-2
3 BaraA
HR = -0.08, p = 0.50
3 Dso
HR = +0.47, p = 7.2e-5
n exp
3 AttC
HR = -0.09, p = 0.46
5 AttD
HR = +0.45, p = 2.5e-5
4 DroAtt
HR = -0.15, p = 0.23
5 DroSK4
HR = -0.32, p = 2.0e-3
8 DptSK1
HR = -0.33, p = 2.4e-3
3 DptAS69R
HR = -0.37, p = 2.8e-3
2 DptA[822]
HR = -0.21, p = 0.16
3 DptB
HR = -0.07, p = 0.60
C
x p < .10 * p < .05 ** p < .01 *** p < .001
Median lifespan (days)
OR-R
iso w1118
Def
Cec
DroSK4
Dro-AttAB
AttC
AttD
Dpt
DptA[822]
DptAS69R
DptB
Drs
Mtk
Dso
BaraA
Bom
mth1
dnr1[2-133]
spz
Rel
ATM8
D
% climbing 5cm in 10s
Days post-eclosion
OR-R
iso w1118
mth1
spz
Rel
ATM8
other AMP mutations

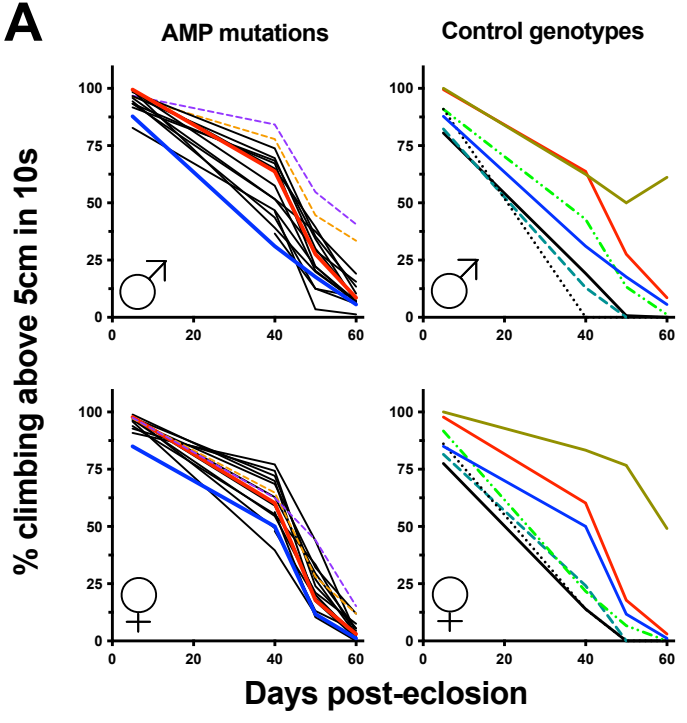

A
n total = 1471
Percent alive
Time (days)
♂+♀
n exp
4 iso w1118
3 Gr A
3 Gr B
3 Gr C
3 Gr D
3 Gr AB
3 Gr AC
3 Gr BC
3 ΔAMP10 (Cec+)
4 ΔAMP14
4 RelE20 (Fig. 2)
C
iso w1118
ΔAMP14
male
female
B
p < .10 p < .05 p < .01 p < .001
Median lifespan (days)
from Fig. 2
iso w1118
DefSK3
RelE20
iso w1118
Gr A
Gr B
Gr C
Gr D
Gr AB
Gr AC
Gr BC
ΔAMP10 (Cec+)
ΔAMP14
D
% climbing 5cm in 10s
overlaid from Fig. 2
mth1
iso w1118
OR-R
Days post-eclosion
iso w1118
GrA
GrB
GrC
GrD
GrAB
GrAC
GrBC
ΔAMP10 (Cec+)
ΔAMP14

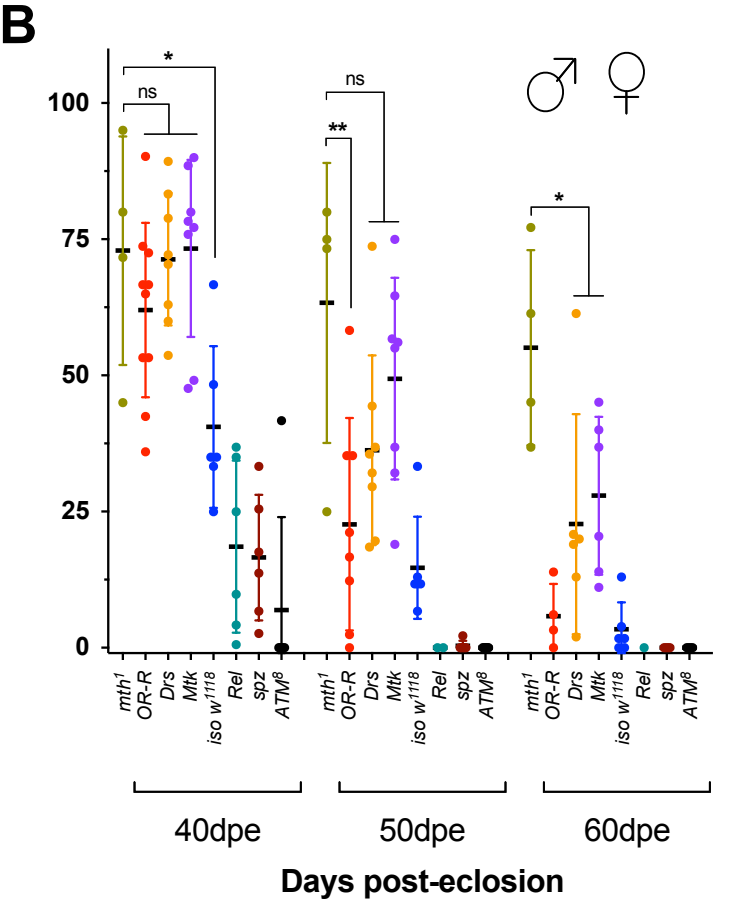

A
Percent alive
Time (days)
0
20
40
60
80
100
0
25
50
75
100
n^{exp}
3 iso w^{1118}
3 ΔAMP14
3 Rel^{E20}
ABX
6 iso w^{1118} (Fig 2)
4 iso w^{1118} (Fig 3)
8 OR-R (Fig. 2)
4 ΔAMP14 (Fig 3)
4 Rel^{E20} (Fig 2)
CR
B
ABX
CR
Median lifespan (days)
.971
.006
.285
**
OR-R CR
iso w^{1118}
ΔAMP14
Rel^{E20}
C
ABX
iso w^{1118}
ΔAMP14
Rel^{E20}
CR
iso w1118 (Fig. 2)
iso w^{1118} (Fig. 3)
ΔAMP14 (Fig. 3)
Rel^{E20} (Fig. 2)
% climbing 5cm in 10s
Days post-eclosion
5
40
50
60


A
Percent alive
100
75
50
25
0
0
10
20
30
40
50
Time (days)
n^exp
3 iso w^1118
3 OR-R
3 dnr1[2-133]
3 mth^1
3 ΔAMP14
3 Rel^E20
B
Median lifespan (days)
40
20
0
ns ns ns ns *** ***
ns * ns *** ***
vs. iso w^1118
vs. OR-R
iso w^1118
OR-R
dnr1[2-133]
mth^1
ΔAMP14
Rel^E20
C
% climbing 5cm in 10s
100
75
50
25
0
5
10
15
20
25
30
Days post-eclosion


A
Percent alive
100
75
50
25
0
0
20
40
60
80
100
Time (days)
♂+♀
Act>OR
Act>Drs-IR
Act>DptA-IR
Act>Mtk-IR
Act>DptB-IR
Act>Def-IR

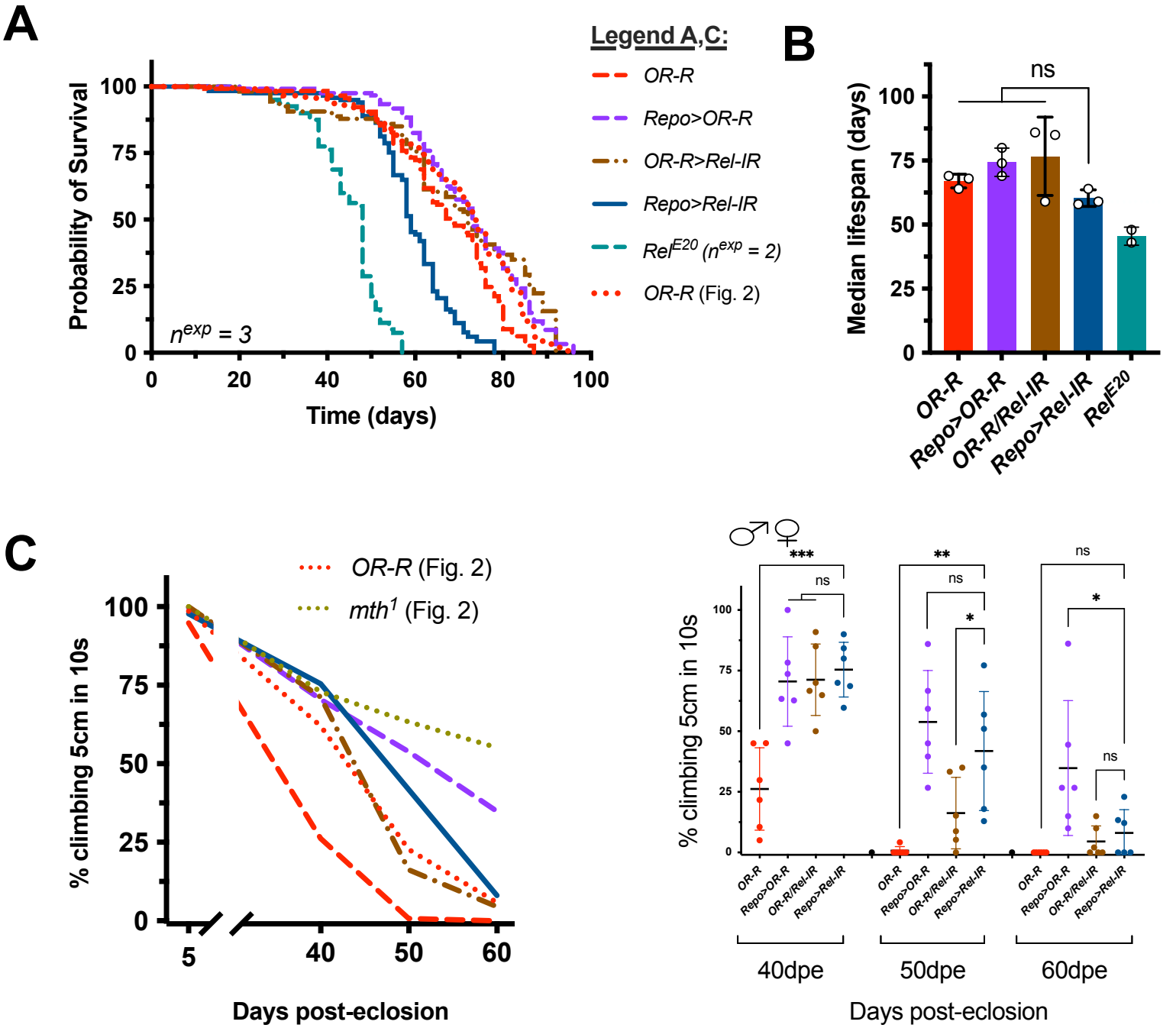

B
Percent alive
100
75
50
25
0
0
20
40
60
80
100
Time (days)
♂+♀
OR-R
OR-R/Def-IR
OR-R/DptA-IR
OR-R/DptB-IR
OR-R/Drs-IR
Act>Drs-IR
OR-R/Mtk-IR
Act>Mtk-IR
OR-R/Repo
Repo>Def-IR
Repo>DptA-IR
Repo>DptB-IR
Repo>Drs-IR
Repo>Mtk-IR





Legend (A):

- *iso w*^{1118}
- *OR-R*
- *Bom*
- *Def*
- *Cec*
- *Drs*
- *Mtk*
- *BaraA*
- *Dso*
- *AttC*
- *AttD*
- *Dro-AttAB*
- *Dro*^{SK4}
- *Dpt*^{SK1}
- *DptA*^{S69R}
- *DptA[822]*
- *DptB*
- *mth*^{1}
- *dnr1[2-133]*
- *spz*
- *Rel*
- *ATM*^{8}


A
Probability of Survival
100
75
50
25
0
n^{exp} = 3
0
20
40
60
80
100
Time (days)
Legend A,C:
OR-R
Repo>OR-R
OR-R>Rel-IR
Repo>Rel-IR
Rel^{E20} (n^{exp} = 2)
OR-R (Fig. 2)
B
Median lifespan (days)
ns
OR-R
Repo>OR-R
OR-R/Rel-IR
Repo>Rel-IR
Rel^{E20}
C
OR-R (Fig. 2)
mth^1 (Fig. 2)
% climbing 5cm in 10s
5
40
50
60
Days post-eclosion
♂ ♀
***
ns
**
*
40dpe
50dpe
60dpe
Days post-eclosion

↑ increases with age ↓ decreases with age ≈ about equal after aging

Wild-type	**Body** *(sans head)*				**Head**			
	DptA	*Drs*	*AttA*	*Dro*	*DptA*	*Drs*	*AttA*	*Dro*
Oregon-R	↑	↑	↑↑↑	↑↑↑	↑	↓	↓↓↓	↓↓↓
Exelexis	↑↑	≈	↑↑	↑↑↑	↑	↑	≈	↑↑
w^{1118} VDRC	↑	↓	↑	≈	↑↑	↓	≈	↑↑
Canton S	≈	≈	↑	≈	≈	≈	≈	↓
Overall trend:	up	no change	up	mixed	up	no change	no change	mixed