Natural and laboratory mutations in *kuzbanian* are associated with heavy metal stress phenotypes in *Drosophila melanogaster*

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ABSTRACT

Organisms must cope with altered environmental conditions such as high concentrations of heavy metals. Stress response to heavy metals is mediated by the metal-responsive transcription factor 1 (MTF-1), which is conserved from Drosophila to humans. MTF-1 binds to metal response elements (MREs) and changes the expression of target genes. *kuzbanian* (*kuz*), a metalloendopeptidase that activates the evolutionary conserved *Notch* signaling pathway, has been identified as an MTF-1 target gene. We have previously identified a putatively adaptive transposable element in the *Drosophila melanogaster* genome, named *FBti0019170,* inserted in a *kuz* intron. In this work, we investigated whether laboratory-induced mutations in *kuz* are associated with zinc stress phenotypes. We found that both embryos and adult flies overexpressing *kuz* are more tolerant to zinc compared with wild-type flies. On the other hand, we found that the effect of *FBti0019170* on zinc stress tolerance depends on developmental stage and genetic background. Moreover, in the majority of the genetic backgrounds analyzed, *FBti0019170* has a deleterious effect in unpolluted environments in pre-adult stages. These results highlight the complexity of natural mutations and suggest that besides laboratory-induced mutations natural mutations need to be studied in order to accurately characterize gene function and evolution.

INTRODUCTION

Heavy metals are non-degradable substances that are natural constituents of the Earth crust[1]. Some heavy metals, such as iron, copper, and zinc, are required at structural and catalytic sites in proteins and are thus vital for many biological processes such as transcription, respiration, and growth[1,2,3]. Indeed, heavy metal deficiencies are related to animal and human diseases such as neurodegenerative and cardiovascular disorders[2,4,5,6]. Although essential heavy metals are necessary for protein activity, when they are present at high concentrations they may bind to inappropriate sites in proteins interfering with their functions. Thus, under limiting conditions essential heavy metals have to be enriched while under excess conditions they have to be removed[7].

Response to heavy metal stress is mediated by the metal-responsive transcription factor-1 (MTF-1). MTF-1 is conserved from insects to vertebrates and besides heavy metal stress it also mediates the response to oxidative stress and hypoxia[8,9,10,11,12]. MTF-1 binds to short DNA sequence motifs known as metal response elements (MREs) activating or repressing expression of target genes[13]. Functional MREs have been located in the promoter regions, downstream of the transcription start site, and in the introns of metal-responsive genes[7,13,14, 15]. In Drosophila, metallothioneins are the best characterized MTF-1 target genes. Metallothioneins have an extremely high affinity for heavy metal ions and play a role in both metal homeostasis and in defense against toxicity of heavy metals[14].

Although their important role, a family knockout of all four Drosophila metallothioneins genes revealed that besides these proteins, other MTF-1 target genes must play a role in response to heavy metals and more specifically in zinc defense[16]. As expected according to these results, a genome-wide screen for MTF-1 target genes identified several candidate genes that respond to

the presence of heavy metals in the environment[15]. One of these candidate genes, *kuzbanian* (*kuz*), is a metalloendopeptidase that controls many biological processes during development and differentiation[17]. *kuz* belongs to the ADAM family of metalloendopeptidases that are zinc-dependent enzymes that have been shown to mediate stress response in mammals[18].

In a previous work, we identified a putatively adaptive natural transposable element (TE), *FBti0019170,* inserted in an intron of *kuz* in *Drosophila melanogaster* natural populations[19] (Fig. 1A). *FBti0019170* is a 4.7 kb non-LTR retrotransposon that belongs to the *F-element* family. *FBti0019170* is a strong candidate to play a role in the out-of-Africa adaptation: while most TEs are deleterious and thus present at low frequencies in populations, *FBti0019170* is present at high frequencies in North American populations and at low frequencies in African populations[19]. Additionally, the regions flanking this insertion showed signatures of a partial selective sweep. This suggests that *FBti0019170* has increased in frequency due to positive selection[19]. *FBti0019170* is located in the center of the sweep and we could not identify any other linked mutation further suggesting that the TE is the causal mutation. We have also already shown using allele-specific expression experiments that a *kuz* allele carrying *FBti0019170* insertion is overexpressed compared to a *kuz* allele without this insertion[19].

In this work, we investigated whether *kuz* is involved in heavy metal stress response, as has been previously suggested[15], and whether *FBti0019170* has fitness consequences for flies that carry this natural insertion. We performed heavy metal stress tolerance assays using zinc chloride. High concentrations of zinc chloride are relevant for *D. melanogaster* natural populations because of its used in pesticides and fertilizers (www.epa.gov). We performed experiments with laboratory mutant flies and with flies collected in natural populations. Furthermore, because tolerance to environmental stress might differ between developmental stages, as has been already

shown for lead, alcohol, and heat stress [20,21,22,23,24], we tested zinc stress tolerance in adult and pre-adult stages.

RESULTS

kuz-overexpressing flies are associated with increased zinc stress tolerance

To check whether *kuz* is involved in zinc stress response, we first determined the concentration of zinc that is required to kill 50% of wild-type flies (LD_{50}). We tested 5 mM, 10 mM, and 20 mM and determined that 20 mM was the adequate dose for both male and female flies (Fig. S1A) (see Material and Methods). We then compared the survival rate of transgenic flies overexpressing *kuz*, *kuz-overexpressing* flies, with wild-type flies with a similar genetic background: *kuz-wildtype* flies (see Material and Methods). In nonstress conditions, that is, flies kept in standard fly food, we found no differences in the survival of *kuz-overexpressing* and *kuz-wildtype* flies (Fig. 2A). However, under zinc stress conditions, we found that *kuz-overexpressing* flies had higher survival than *kuz-wildtype* flies for both males and females (Fig. 2A and Table 1). To confirm these results, we performed a replicate of the experiment using flies from the same two strains a few generations later. We obtained the same results: both *kuz-overexpressing* male and female flies had higher survival than *kuz-wildtype* flies under zinc stress conditions (Fig. 2B and Table 1).

Overall, we found that while there were no differences in survival between *kuz-overexpressing* and *kuz-wildtype* flies in nonstress conditions, *kuz-overexpressing* flies had higher survival than *kuz-wildtype* flies under zinc stress conditions (Fig.2 and Table 1). These results suggest that *kuz* could play a role in response to zinc stress.

Egg to adult viability is higher in *kuz-overexpressing* flies in zinc stress conditions

As mentioned above, tolerance to environmental stress might differ between developmental stages. Thus, we also tested egg to adult viability under zinc stress conditions in *kuz-overexpressing* and *kuz-wildtype* flies. We first performed an LD_{50} and found that 10 mM zinc is the dose at which ~50% of the wild-type embryos do not emerge (Fig. S1B) (see Material and Methods).

We compared the egg to adult viability of *kuz-overexpressing* flies with *kuz-wildtype* flies in nonstress and in zinc stress conditions (Fig. 3). ANOVA analysis showed that the experimental condition (nonstress or zinc stress) and the interaction between experimental condition and genotype (*kuz-overexpressing* or *kuz-wildtype*) were significant (Fig. 3 and Table 2): *kuz-overexpressing* flies had higher egg to adult viability than *kuz-wildtype* flies in zinc stress conditions. This suggested that *kuz* could play a role in zinc stress response also in pre-adult developmental stages.

FBti0019170 is associated with increased zinc tolerance in flies from outbred populations

As mentioned above, *FBti0019170*, inserted in the third intron of *kuz*, shows signatures of a selective sweep suggesting that this natural insertion has increased in frequency due to positive selection. We thus investigate whether flies with *FBti0019170* were associated with increased tolerance to zinc stress. We analyzed both adult fly survival and egg to adult viability in nonstress and zinc stress conditions in natural strains with different genetic backgrounds: outbred

strains and isofemale strains. Analyzing different genetic backgrounds is needed because the effect of mutations is often background dependent[25].

We first constructed two outbred laboratory strains: one outbred strain homozygous for the presence of *FBti0019170* and one outbred strain homozygous for the absence of this insertion (see Material and Methods). We subjected these two outbred strains to zinc stress and we found that both male and female flies with *FBti0019170* had higher survival than flies without *FBti0019170* (Fig.4A, Table 1). The same results were obtained with the same outbred strains analyzed a few generations later (Fig.4B and Table 1). In both replicas, no differences in survival between flies with and without *FBti0019170* were found in nonstress conditions (Fig. 4). Overall, we found that *FBti0019170* is associated with increased zinc tolerance in adult flies.

FBti0019170 is associated with decreased egg to adult viability both in nonstress and in zinc stress conditions in outbred populations

We also tested whether embryos from the outbred strain with *FBti0019170* insertion were more tolerant to zinc stress compared with embryos from the outbred strain without the insertion. We performed two replicas of the experiment (Fig. 5). ANOVA analysis showed that the experimental condition (nonstress vs zinc stress) and the insertion genotype (presence vs absence of *FBti0019170*) were significant (Table 2). Both in nonstress and zinc stress conditions, flies with *FBti0019170* had a lower survival rate compared to flies without the insertion (Fig. 5). Thus, while *FBti0019170* is associated with higher adult survival in zinc stress conditions, it is also associated with lower egg to adult viability both in nonstress and in zinc stress conditions.

157 **_FBti0019170_ effect in adult survival in isofemale strains depends on the genetic**
158 **background**

159 We also performed adult survival experiments in nonstress and zinc stress conditions with
160 different isofemale strains. Adult survival of *IV22*, *IV145*, and *B45* isofemale strains containing
161 *FBti0019170* insertion was compared with the adult survival of *B47* isofemale strain without this
162 insertion (see Material and Methods). No differences in survival between flies with and without
163 *FBti0019170* insertion were observed in nonstress conditions. However, we found that *IV22* flies
164 with *FBti0019170* had lower survival than *B47* flies without *FBti0019170* (Fig. 6A Table 1). We
165 confirmed these results by performing a second replica a few generations later (Fig. 6B; Table
166 1). Similarly, *IV145* flies with *FBti0019170* also had lower survival than *B47* flies without
167 *FBti0019170* (Fig. 6C Table 1). On the other hand, male flies of *B45* strain had higher survival
168 than male flies of *B47* strain (Fig. 6D; Table 1) while *B45* female flies had higher survival than
169 *B47* female flies at early time points while they had lower survival at later time points (Fig. 6D;
170 Table 1).
171 Therefore, in isofemale strains, the association between *FBti0019170* and heavy metal stress
172 phenotypes depends on the background: while *IV22* and *IV145* isofemale strains with the
173 insertion were more sensitive to zinc stress, *B45* were more tolerant to zinc stress in early time
174 points compared to flies without this insertion.
175

176 **_FBti0019170_ effect in egg to adult viability in isofemale strains also depends on the genetic**
177 **background**

178 We also checked egg to adult viability in the isofemale strain *IV22* that contains *FBti0019170*
179 insertion and in *B47* strain without this insertion. Additionally, we identified two strains

heterozygous for *FBti0019170*, *B38* and *IV52*, and we generated homozygous flies for the presence and homozygous flies for the absence of *FBti0019170* by brother-sister crosses (see Material and Methods). Note that flies with and without the insertion generated from heterozygous strains, have more similar genetic backgrounds compared with isofemale strains with and without the insertion.

For *IV22* and *B47*, we found that the experimental condition, the insertion genotype and the interaction between these two factors were significant (Figure 7A and Table 2). *IV22* flies that contain *FBti0019170* insertion had lower viability in nonstress conditions and higher viability in zinc stress conditions than flies without *FBti0019170.* For the *B38* flies with and without *FBti0019170* insertion, we found that the experimental condition, and the interaction between experimental condition and insertion genotype were significant (Fig. 7B; Table 2): in nonstress conditions flies with *FBti0019170* had lower viability while in zinc stress conditions had higher viability than flies without this insertion. Finally, for *IV52* strains with and without *FBti0019170*, only the experimental condition was significant (Fig. 7C; Table 2).

Overall, we found that the effect of *FBti0019170* on egg to adult viability depends on the genetic background: in two of the three backgrounds analyzed the presence/absence of *FBti0019170* and/or the interaction between the genotype and the experimental condition was significant (Fig. 7).

FBti0019170* could add a metal-responsive element to the intron of *kuzbanian

As mentioned above, *kuz* was found to be a MTF-1 target gene, and we have shown that *kuz-overexpressing* flies were more zinc tolerant compared to *kuz-wildtype* flies. We have also found that flies with *FBti0019170* were associated with increased zinc tolerance in some genetic

backgrounds. To shed light on the mechanisms underlying zinc stress response of laboratory-induced and natural *kuz* mutations, we investigated whether *kuz* has metal responsive elements (MREs) in its promoter region and whether *FBti0019170* is introducing any additional MRE. We could not detect any MRE in the *kuz* promoter. On the other hand, we found a high score MRE nearby the 3' end of *FBti0019170* (Fig. 1C and supplementary Table S2). This prompted us to investigate whether there were other MREs in the *kuz* intron where *FBti0019170* is inserted, and we identified three additional MREs (Fig. 1C). Interestingly, two of these MREs are located only 462 bp downstream of the MRE introduced by *FBti0019170* while the third intronic MRE is located nearby the 3' end of the intron (Fig. 1C and Table S2).

Overall, we found four MREs present in the *kuz* intron where *FBti0019170* is inserted. *FBti0019170* adds one of these four MREs. Because there is a correlation between the number of transcription factor binding sites and the increase in the level of expression of nearby genes[26], these results suggest that *FBti0019170* could affect the expression of *kuz* and thus could play a role in zinc stress response.

Flies homozygous for the presence and for the absence of *FBti0019170* insertion do not show differences in the level of *kuz* expression

We checked whether outbred flies homozygous for the presence of *FBti0019170* showed different levels of *kuz* expression compared to outbred flies without this insertion. We performed qRT-PCR experiments both in nonstress and in zinc stress conditions for both male and female flies. No differences in the level of expression of *kuz* between flies with and without *FBti0019170* were found in nonstress or zinc stress conditions for males or for females (Fig. 8).

DISCUSSION

In this work, we showed that a laboratory-induced mutation in *kuz* is associated with tolerance to zinc stress both in adult (Fig. 2) and embryo stages (Fig. 3). These results are consistent with a role of *kuz* in heavy metal stress response, as it has been previously suggested by experiments performed with MTF-1 mutant flies that identified *kuz* as a candidate heavy metal-responsive gene[15]. *kuz, a* metalloprotease that belongs to the ADAM family, is a component of the Notch signaling pathway that plays a role in axon guidance in the developing central nervous system[17,27-30]. ADAM metalloproteases in mammals, and more specifically *kuz* ortholog ADAM10, ADAM17, and to a lesser extend ADAM9, also regulate epidermal growth factor receptor (EGFR) activation in response to a variety of stress agents[18]. Stress-induced EGFR activation leads to the activation of mitogen-activated protein kinase (MAPKs) signaling that trigger transcriptional regulation of a variety of stress-response genes[1]. Thus, both *kuz* and its ortholog gene ADAM10 could be involved in response to stress. Indeed, the organomercurial compound p-aminophenylmercuric acetate (APMA) have been reported to upregulate both *kuz* and *ADAM10* protease activity[31,32] and methylmercury has been suggested to activate ADAM proteases in Drosophila[33].

Our previous results showing that *FBti0019170*, which is inserted in the third intron of *kuz,* has most probably increased in frequency due to positive selection, prompted us to investigate whether flies with this insertion have increased zinc tolerance. To test this hypothesis, we generated an outbred population and analyzed five isofemale strains established from two

different natural populations. We found that the effect of *FBti0019170* on egg to adult viability and on adult survival under zinc stress conditions depended on the genetic background analyzed (Figure 4-7). In four of the six backgrounds analyzed, the presence of *FBti0019170* insertion was associated with higher adult survival or increased egg to adult viability under zinc stress conditions. In the other two backgrounds, *FBti0019170* was not significantly associated with zinc stress phenotypes or *FBti0019170* was associated with increased sensitivity to zinc stress (Fig. 4-7). These results are consistent with previous experimental data showing that the mutational effects in one genetic background are often enhanced or suppressed in other backgrounds[34,35]. For example, different mutations in the same gene have been associated with both increased and decreased life span in *D. melanogaster*[36]. Different effects of a mutation, as the ones described in this work, are most likely explained by epistatic interactions in the different genetic backgrounds[25].

Our results also showed that *FBti0019170* was associated with lower egg to adult viability in nonstress conditions in three of the four backgrounds analyzed (Fig. 5 and 7). Between-environments trade-offs have been reported for cadmium resistance in *D. melanogaster*[37] as well as for other environmental stress conditions such as oxidative stress[38]. Two mechanisms have been proposed to explain the fitness costs of heavy-metal resistance in unpolluted environments: the activation of detoxification enzymes might use resources that are then unavailable for other fitness traits, and/or resistant flies might be less efficient at metal uptake or utilization, which would lead to micronutrient deficiencies[37]. In the case of *FBti0019170*, the deleterious effect of the mutation was found in egg to adult viability while no cost of selection was found in adult stages. As mentioned above, *kuz* plays a role in development and differentiation[17,27-30]. Thus, it

could be that the cost of selection of *FBti0019170* is related to the role of *kuz* during development.

Consistent with the activation of *kuz* by zinc, we found *in silico* evidence for three MREs located in the *kuz* intron where the candidate adaptive TE *FBti0019170* is inserted (Fig. 1C). Indeed, *FBti0019170* insertion adds another MRE 462 bp upstream of the three intronic MREs (Fig. 1C). We thus check the expression of *kuz in* flies homozygous for the presence and for the absence of *FBti0019170* using qRT-PCR. We did not find differences in *kuz* expression in nonstress or in zinc stress conditions (Fig. 7). However, we have previously shown, using allele-specific expression, that an allele carrying *FBti0019170* insertion is overexpressed compared to an allele that does not carry this insertion[19]. Because allele-specific expression is performed in F_1 hybrids in which the two alleles share the same cellular environment, the expression differences between the two alleles must be due to cis-regulatory differences[39]. *FBti0019170* is thus a strong candidate to be responsible for the observed differences in *kuz* expression level[19]. The lack of differential expression in homozygous flies could be partly explained by the higher sensitivity of allele-specific expression experiments compared to qRT-PCR[40]. Besides, it could be that *FBti0019170* effect on *kuz* expression is overdominant as has been described for a few genes involved in temperature stress response[41]. Further experiments are needed in order to understand the molecular mechanism underpinning *FBti0019170* insertion effects.

As with other quantitative traits, including starvation stress and olfactory behavior, we have found that mutations in a gene with well-characterized roles in development affect tolerance to zinc stress[34]. Our results showed that while *kuz* laboratory-induced mutations are consistently

associated with increased tolerance to heavy metal stress in embryo and adult stages, natural mutations have more complex fitness effects that depend on the developmental stage and the genetic background. Furthermore, while no cost of selection was associated with the laboratory-induced mutation, we found that *FBti0019170* is associated with decreased egg to adult viability in unpolluted environments. Different fitness effects of laboratory-induced and natural mutations have previously been described suggesting that the analysis of natural mutations is needed in order to accurately characterize gene function and evolution[42,43].

METHODS

Genotyping flies for presence/absence of *FBti0019170*.

To check the insertion genotype, that is, whether different fly stocks were homozygous for the presence, homozygous for the absence, or heterozygous for *FBti0019170* insertion, we performed PCR with two pairs of primers ([19]. Primer pair *Left* (L) and *Right* (R) were designed to check for the presence of *FBti0019170* (Fig.1B). The *Left* primer (TTCGGAGTGAAAACATCCAAAGA) binds to *FBti0019170* while the *Right* primer (TTGAATATTGTGTCGATTGCGTG) binds to the downstream sequence flanking the insertion (Fig.1B). This primer pair only gives a PCR band when *FBti0019170* is present. On the other hand, primer pair *Flanking* and *Right* was designed to check for the absence of *FBti0019170*. The *Flanking* (FL) primer (GACGAATTCATAAATTGGCGGTT) binds to the upstream sequence flanking the insertion (Fig.1B). This primer pair only gives a PCR band when *FBti0019170* is absent. If only the *Left-Right* primer pair gives a PCR band, the strain is homozygous for the presence of *FBti0019170*. If only the *Flanking-Right* primer pair gives a

317 PCR band, the strain is homozygous for the absence of *FBti0019170*. Finally, if both primers
318 give PCR bands, the strain is heterozygous for *FBti0019170* insertion[44].
319 48 different isofemale strains collected in Stockholm (Sweden, "B" strains) and 15 isofemale
320 strains collected in Bari (Italy, "IV" strains), available in our laboratory, have been tested by
321 PCR to check for the presence/absence of *FBti0019170* natural insertion (Table S1).
322
323 **Fly strains**
324 **Laboratory mutant strains**. We used transgenic flies that carry a full copy of *kuz* coding region
325 under the control of a *UAS* promoter[45] (Bloomington stock # 5816). To activate the expression of
326 *kuz*, we crossed the flies with transgenic flies that carry the *GAL4* gene under the control of
327 *Act5C* promoter (Bloomington stock # 4414) and we kept the crosses at 25ºC. A total of 200
328 virgin female of *kuz* mutant flies were crossed with 200 male of *Act5C-GAL4* flies. F_1 flies
329 carrying *UAS-kuz* and *Act5C-GAL4*, and thus overexpressing *kuz*, have wild-type wings (*kuz-*
330 *overexpressing* flies) while F_1 flies that do not have the *Act5C-GAL4* chromosome and thus do
331 not over-express *kuz* have *Curly* wings. A stock with a w[*] genetic background, as the *kuz*
332 transgenic flies background, was used as the baseline for the experiment *(kuz-wildtype* flies;
333 Bloomington stock # 7087).
334 **Outbred populations**. To create an outbred population with *FBti0019170* insertion and an
335 outbred population without the insertion, a total of 10 isofemale strains were selected: five
336 strains homozygous for the presence of the element (*B7, B45, IV33, IV49, IV50*) and five strains
337 homozygous for the absence (*B2, B4, B8, B15, B18*) (Table S1). We collected 10 virgin females
338 and 10 males from each one of these strains. We did two crosses by mixing males and females
339 with the TE to create an outbred *FBti0019170 (+)* strain, and males and females without the TE

to create an outbred *FBti0019170 (-)* strain. We kept the two populations for at least seven generations before performing any phenotypic experiments.

Isofemale Strains. We selected three strains in which *FBti0019170* was present (*IV22*, *IV145* and *B45*) and one strain in which *FBti0019170* was absent (*B47*) to perform phenotypic experiments (Table S1). Isofemale flies heterozygous for *FBti0019170* insertion were also selected to create homozygous flies for the presence and homozygous flies for the absence of *FBti0019170* (see below).

Heterozygous strains. We first identified two isofemale strains (*B38* and *IV52*) that were heterozygous for *FBti0019170* insertion. We then collected 10 to 25 virgin females from each strain and crossed them individually with males from the same strain. F_1 progeny of all the crosses were checked for the presence/absence of the *FBti0019170* insertion by PCR. Brother-sister crosses were performed until we obtained flies that were homozygous for the presence of *FBti0019170* and flies that were homozygous for the absence of *FBti0019170*. Those flies were amplified for several generations in order to obtain enough quantity of flies to perform the experiments.

Zinc stress experiments

We used zinc chloride ($ZnCl_2$) as a heavy metal stress agent (Sigma-Aldrich catalog # Z0152). We have performed zinc stress experiments in two different life stages: adult flies and embryos.

Adult flies. To determine the Lethal $Dose_{50}$ (LD_{50}) for the adult flies experiments, we tested three different zinc concentrations: 5 mM, 10 mM and 20 mM. The experiments allowed us to identify the $ZnCl_2$ concentration at which about 50% of the adult flies die. $ZnCl_2$ was dissolved in water and added to the fly food to the desired final concentration. Standard fly food was used

for the nonstress conditions. We used the outbred population without *FBti0019170* to establish the LD_{50}. We analyzed 10 vials for each concentration and sex with 20 five to seven day-old flies each.

For the zinc stress tolerance experiments with adult flies from natural populations, a total of 100 vials with 20 flies each were used including 40 vials for nonstress conditions (10 vials per sex and per strain) and 60 vials for zinc stress condition (15 vials per sex and per strain). We used five to seven day-old flies.

For the zinc stress experiments performed with *kuz-overexpressing* flies, we used 40 vials for the nonstress condition and 60 vials for the zinc stress condition. 10 vials per condition were used to perform the experiments for the *kuz-wildtype* flies. We used five to seven day-old flies.

Embryos. We determined the LD_{50} using embryos from an isofemale strain without *FBti0019170* insertion (*B47*). These experiments allowed us to identify the $ZnCl_2$ concentration at which about 50% of the embryos do not emerge. We first tested three different $ZnCl_2$ concentrations: 1.25 mM, 2.5 mM and 5 mM. The same strain and protocol but different concentrations of $ZnCl_2$ were tested in a second LD_{50} experiment: 5 mM, 10mM, 20 mM. In both cases, standard fly food was used for the nonstress conditions. Five day-old isofemale flies without *FBti0019170* insertion were kept in chambers with agar and apple juice plates to lay eggs during 4 hours. 10 vials with 50 embryos each were analyzed for each of the three $ZnCl_2$ concentrations and for the nonstress condition.

Once the LD_{50} was determined, we performed zinc stress experiments using 50 embryos per vial. For the *kuz-wildtype* strain, we analyzed 10 vials for nonstress and 10 vials for zinc stress

conditions. For the *Kuz-overexpressing* strains, we analyzed 20 vials for nonstress and 20 vials for zinc stress conditions. For natural strains, we used 15 vials for the zinc stress condition and 10 vials for the nonstress condition.

In silico prediction of MTF-1 binding sites

We use TFBSTools software[46] to predict binding sites of MTF-1 in the *kuz* promoter region and in the *kuz* intron where *FBti0019170* is inserted. Position weight matrices for MTF-1 transcription factor were obtained from JASPAR database[47]. The PB0044.1 and PB00148.1 matrices were used. Although the default score threshold in TFBSTools is 0.75, we were conservative and we only considered significant those hits with a score threshold ≥ 0.95. We used the release 6.02 of the *D. melanogaster* genome available at http://flybase.org.

qRT-PCR Expression analysis

We quantified the expression of *kuz* in nonstress and stress conditions induced by zinc. Five day-old outbred flies (30 females and 50 males) were separated by sex and transferred to standard fly food as well as food containing 20 mM zinc for 48 hours before freezing them in liquid nitrogen. We did three biological replicas with flies from three different generations for each sex and condition. We purified total RNA using Trizol reagent and we synthesized cDNA using 1µg of RNA after treatment with DNase. We then used the cDNA for quantitative PCR analysis using *Act5C* as a housekeeping gene. The primers used were as follows: *kuz_left primer*: CACCGAGCATCGCAACATAC, *kuz_right primer*: GAATTGCGACAGGCCGAATC, *Act5C_left primer*: ATGTCACGGACGATTTCACG, and *Act5C_right primer*: GCGCCCTTACTCTTTCACCA.

Results were analyzed using the dCT method and following the recommendations of the MIQE guideline[48].

Statistical analysis

Log-rank test. The number of surviving flies for both nonstress and stress conditions were counted every 24 hours for five consecutive days. We used Kaplan-Meier to estimate the survival functions and performed a log-rank test to compare the functions between flies with and without the insertion using the SPSS software.

The odds-ratio (O.R.) was calculated as: (number of tolerant flies alive/number of tolerant flies dead)/ (number of sensitive flies alive/number of sensitive flies dead). The upper and lower 95% O.R. confidence interval was calculated as: e ^ [ln OR ± 1.96 √ (1/ number of tolerant flies alive + 1/ number of tolerant flies dead + 1/ number of sensitive flies alive + 1/ number of sensitive flies dead)]. We used the 95% confidence interval as a proxy for the presence of statistical significance when it does not overlap the null value, that is, O.R. = 1.

Two-way ANOVA analyses. The number of flies emerging from the experiments performed with embryos was transformed to a uniform distribution using the rank transformation. SPSS v21 was used to perform the ANOVA analyses. Two different variables were considered for the ANOVA analyses: the genotype (*kuz-overexpressing/ kuz-wildtype* or *FBti0019170* present/ *FBti0019170* absent), and the experimental condition (nonstress and zinc stress). The replicate effect was also considered. As a measure of the effect size, we estimated partial eta-squared values (0.01 small effect, 0.06 medium effect, and 0.14 large effect).

REFERENCES

1 Straalen, N. M. v., Roelofs, D. An introduction to ecological genomics. 363 p (Oxford University Press,, Oxford, 2012).

2 Lichtlen, P. & Schaffner, W. Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. *Bioessays* **23**, 1010-1017 (2001).

3 Janssens, T. K. S., Roelofs, D. and Van Straalen, N. M. Molecular mechanisms of heavy metal tolerance and evolution in invertebrates. *Insect Science* **16**, 3-18 (2009).

4 Nielsen, F. H. History of zinc in agriculture. *Adv Nutr* **3**, 783-789 (2012).

5 Kaur, K., Gupta, R., Saraf, S. A. and Saraf, S. K. Zinc: The Metal of Life. *Comprehensive Reviews in Food Science and Food Safety* **13**, 358-376 (2014).

6 Tyszka-Czochara, M. *et al.* The role of zinc in the pathogenesis and treatment of central nervous system (CNS) diseases. Implications of zinc homeostasis for proper CNS function. *Acta Pol Pharm* **71**, 369-377 (2014).

7 Gunther, V., Lindert, U. & Schaffner, W. The taste of heavy metals: gene regulation by MTF-1. *Biochim Biophys Acta* **1823**, 1416-1425 (2012).

8 Zhang, B., Egli, D., Georgiev, O. & Schaffner, W. The Drosophila homolog of mammalian zinc finger factor MTF-1 activates transcription in response to heavy metals. *Mol Cell Biol* **21**, 4505-4514 (2001).

9 Westin, G. & Schaffner, W. A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene. *EMBO J* **7**, 3763-3770 (1988).

10 Brugnera, E. *et al.* Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucleic Acids Res* **22**, 3167-3173 (1994).

11 Auf der Maur, A., Belser, T., Elgar, G., Georgiev, O. & Schaffner, W. Characterization of the transcription factor MTF-1 from the Japanese pufferfish (*Fugu rubripes*) reveals evolutionary conservation of heavy metal stress response. *Biol Chem* **380**, 175-185 (1999).

12 Balamurugan, K. *et al.* Metal-responsive transcription factor (MTF-1) and heavy metal stress response in Drosophila and mammalian cells: a functional comparison. *Biol Chem* **385**, 597-603 (2004).

13 Stuart, G. W., Searle, P. F., Chen, H. Y., Brinster, R. L. & Palmiter, R. D. A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc Natl Acad Sci U S A* **81**, 7318-7322 (1984).

14 Egli, D. *et al.* Knockout of 'metal-responsive transcription factor' MTF-1 in Drosophila by homologous recombination reveals its central role in heavy metal homeostasis. *EMBO J* **22**, 100-108 (2003).

15 Yepiskoposyan, H. *et al.* Transcriptome response to heavy metal stress in Drosophila reveals a new zinc transporter that confers resistance to zinc. *Nucleic Acids Res* **34**, 4866-4877 (2006).

16 Egli, D. *et al.* A family knockout of all four Drosophila metallothioneins reveals a central role in copper homeostasis and detoxification. *Mol Cell Biol* **26**, 2286-2296 (2006).

17 Meyer, H. *et al.* Drosophila metalloproteases in development and differentiation: the role of ADAM proteins and their relatives. *Eur J Cell Biol* **90**, 770-778 (2011).

18 Fischer, O. M., Hart, S., Gschwind, A., Prenzel, N. & Ullrich, A. Oxidative and osmotic stress signaling in tumor cells is mediated by ADAM proteases and heparin-binding epidermal growth factor. *Mol Cell Biol* **24**, 5172-5183 (2004).

19 Gonzalez, J., Lenkov, K., Lipatov, M., Macpherson, J. M. & Petrov, D. A. High rate of recent transposable element-induced adaptation in *Drosophila melanogaster*. *PLoS Biol* **6**, e251:0060251 (2008).

20 Loeschcke, V. & Krebs, R. A. Selection for Heat-Shock Resistance in Larval and in Adult *Drosophila buzzatii*: Comparing Direct and Indirect Responses. *Evolution* **50**, 2354-2359 (1996).

21 Gilchrist, G. W., Huey, R. B. & Partridge, L. Thermal sensitivity of *Drosophila melanogaster*: evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. *Physiol Zool* **70**, 403-414 (1997).

22 Malherbe, Y., Kamping, A., van Delden, W. & van de Zande, L. ADH enzyme activity and Adh gene expression in *Drosophila melanogaster* lines differentially selected for increased alcohol tolerance. *J Evol Biol* **18**, 811-819 (2005).

23 Sgro, C. M. *et al.* A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from eastern Australia. *J Evol Biol* **23**, 2484-2493 (2010).

24 van Heerwaarden, B., Lee, R. F., Wegener, B., Weeks, A. R. & Sgro, C. M. Complex patterns of local adaptation in heat tolerance in *Drosophila simulans* from eastern Australia. *J Evol Biol* **25**, 1765-1778 (2012).

25 Chandler, C. H., Chari, S. & Dworkin, I. Does your gene need a background check? How genetic background impacts the analysis of mutations, genes, and evolution. *Trends Genet* **29**, 358-366 (2013).

26 Xie, D. *et al.* Rewirable gene regulatory networks in the preimplantation embryonic development of three mammalian species. *Genome Res* **20**, 804-815 (2010).

27 Sotillos, S., Roch, F. & Campuzano, S. The metalloprotease-disintegrin Kuzbanian participates in Notch activation during growth and patterning of Drosophila imaginal discs. *Development* **124**, 4769-4779 (1997).

28 Lieber, T., Kidd, S. & Young, M. W. kuzbanian-mediated cleavage of Drosophila Notch. *Genes Dev* **16**, 209-221 (2002).

29 Coleman, H. A., Labrador, J. P., Chance, R. K. & Bashaw, G. J. The Adam family metalloprotease Kuzbanian regulates the cleavage of the roundabout receptor to control axon repulsion at the midline. *Development* **137**, 2417-2426 (2010).

30 McFarlane, S. Metalloproteases: carving out a role in axon guidance. *Neuron* **37**, 559-562 (2003).

31 Bland, C. E., Kimberly, P. & Rand, M. D. Notch-induced proteolysis and nuclear localization of the Delta ligand. *J Biol Chem* **278**, 13607-13610 (2003).

32 Sanderson, M. P. *et al.* ADAM10 mediates ectodomain shedding of the betacellulin precursor activated by p-aminophenylmercuric acetate and extracellular calcium influx. *J Biol Chem* **280**, 1826-1837 (2005).

33 Bland, C. & Rand, M. D. Methylmercury induces activation of Notch signaling. *Neurotoxicology* **27**, 982-991 (2006).

34 Mackay, T. F. Mutations and quantitative genetic variation: lessons from Drosophila. *Philos Trans R Soc Lond B Biol Sci* **365**, 1229-1239 (2010).

35 Mackay, T. F. Epistasis and quantitative traits: using model organisms to study gene-gene interactions. *Nat Rev Genet* **15**, 22-33 (2014).

36 Magwire, M. M. *et al.* Quantitative and molecular genetic analyses of mutations increasing Drosophila life span. *PLoS Genet* **6**, e1001037 (2010).

37 Mark, D. F. S. & Sibly, R. M. Genetic Basis of a between-Environment Trade-off Involving Resistance to Cadmium in *Drosophila melanogaster*. *Evolution* **53**, 826-836 (1999).

38 Guio, L., Barron, M. G. & Gonzalez, J. The transposable element *Bari-Jheh* mediates oxidative stress response in Drosophila. *Mol Ecol* **23**, 2020-2030 (2014).

39 Wittkopp, P. J., Haerum, B. K. & Clark, A. G. Evolutionary changes in cis and trans gene regulation. *Nature* **430**, 85-88 (2004).

40 Schaart, J. G., Mehli, L. & Schouten, H. J. Quantification of allele-specific expression of a gene encoding strawberry polygalacturonase-inhibiting protein (PGIP) using Pyrosequencing. *Plant J* **41**, 493-500 (2005).

41 Chen, J., Nolte, V. & Schlötterer, C. Temperature Stress Mediates Decanalization and Dominance of Gene Expression in *Drosophila melanogaster*. *PLoS Genet* **11**, e1004883 (2015).

42 Ullastres, A., Petit, N. & Gonzalez, J. Exploring the Phenotypic Space and the Evolutionary History of a Natural Mutation in *Drosophila melanogaster*. *Mol Biol Evol* **32**, 1800-1814 (2015).

43 Landry, C. R. & Rifkin, S. A. The genotype-phenotype maps of systems biology and quantitative genetics: distinct and complementary. *Adv Exp Med Biol* **751**, 371-398 (2012).

44 Gonzalez, J. & Petrov, D. A. The adaptive role of transposable elements in the Drosophila genome. *Gene* **448**, 124-133 (2009).

45 Fambrough, D., Pan, D., Rubin, G. M. & Goodman, C. S. The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in Drosophila. *Proc Natl Acad Sci U S A* **93**, 13233-13238 (1996).

46 TFBSTools: Software package for transcription factor binding site (TFBS) analysis. R package version 1.6.0, http://jaspar.genereg.net/. v. 1.6.0 (2015).

47 Mathelier, A. *et al.* JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic Acids Res* **42**, D142-147 (2014).

48 Bustin, S. A. *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**, 611-622 (2009).

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AUTHOR CONTRIBUTIONS

H.L.M. performed research, analyzed data, and draft the manuscript. L.G., M.M., Q.R and M.G.B, performed research and analyzed data. J.G. designed research, analyzed data, and wrote the manuscript.

ADDITIONAL INFORMATION

Competing financial interests

The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1. *FBti0019170* is a full-length *F-element*, 4,696 bp, inserted in the third intron of *kuzbanian*.

(A) *kuzbanian* (*kuz*) gene region. White boxes represent UTRs, black boxes represent exons, black lines represent introns and intergenic regions, and the red box represents the *FBti0019170* insertion. (B) The region amplified represents *FBti0019170* insertion (2L: 13,560,515-13,565,210) and its flanking regions. Black arrows show the approximate localization of the three primers designed to check for the presence/absence of *FBti0019170*. (C) Predicted Metal Response Elements are represented in purple: one is located inside *FBti0019170* insertion and the other three in *kuz*'s third intron (see Table S2).

Figure 2. *kuz-overexpressing* flies are associated with zinc stress tolerance.

Survival curves under nonstress (discontinuous lines) and under zinc stress (continuous lines) conditions are represented in purple for *kuz-overexpressing* flies, and in green for *kuz-wildtype*

590 flies. The first replica (A) and the second replica (B) showed that *kuz-overexpressing* flies are
591 more tolerant to zinc stress both in males and in female flies. Each data point in the survival
592 curves represent the average survival for 15 tubes containing 20 flies each for zinc stress
593 conditions and 10 tubes containing 20 flies each for nonstress conditions. In each data point,
594 error bars represent the standard error of the mean (SEM).
595
596 **Figure 3. *kuz-overexpressing* embryos have a higher egg to adult viability under zinc stress.**
597 Each column represents the average of egg to adult viability. *kuz-overexpressing* flies are
598 represented in purple and *kuz-wildtype* flies are represented in green. In each data point, error
599 bars represent the standard error of the mean (SEM).
600
601 **Figure 4. Outbred flies with *FBti0019170* insertion are associated with zinc stress tolerance.**
602 Survival curves under non-stress conditions (discontinuous lines), and under zinc stress
603 (continuous lines) are represented in red for outbred flies with *FBti0019170* insertion, and in
604 black for outbred flies without the insertion. The first (A) and the second replica (B) showed the
605 same results for both males and females. Each data point in the survival curves represent the
606 average survival for 15 tubes containing 20 flies each for zinc stress conditions and 10 tubes
607 containing 20 flies each for nonstress conditions. In each data point, error bars represent the
608 standard error of the mean (SEM).
609
610 **Figure 5. Egg to adult viability in outbred flies with *FBti0019170* is lower than in outbred**
611 **flies without *FBti0019170* in nonstress and in zinc stress conditions.**

612 Each column represents the average of egg to adult viability of 15 vials for zinc stress conditions
613 and 10 vials for nonstress conditions. Outbred flies without the insertion are represented in black
614 and outbred flies with the insertion are represented in red.

615

616

617 **Figure 6. Isofemale strains with *FBti0019170* insertion are more sensitive or more tolerant**
618 **to zinc stress depending on the genetic background.**

619 Survival curves under non-stress conditions (discontinuous lines) and under zinc stress
620 (continuous lines) are represented in red for flies with *FBti0019170* insertion, and in black for
621 flies without the insertion. (A) Survival curves for *IV22* vs *B47* (first replicate), (B) Survival
622 curves for *IV22* vs *B47* (second replicate), (C) survival curves for *IV145* vs *B47,* and (D) survival
623 curves for *B45* vs *B47.* Each data point in the survival curves represent the average survival for
624 15 tubes containing 20 flies each for zinc stress conditions and 10 tubes containing 20 flies each
625 for nonstress conditions. Error bars represent the standard error of the mean (SEM) for each
626 datapoint.

627

628 **Figure 7. Egg to adult viability in isofemale strains with and without *FBti0019170* depends**
629 **on the genetic background**

630 Each column represents the average of egg to adult viability of 15 vials for zinc stress conditions
631 and 10 vials for nonstress conditions. Strains without the insertion are represented in black and
632 strains with the insertion are represented in red. (A) Egg to adult viability in *IV22* flies with
633 *FBti0019170* and B47 flies without *FBti0019170,* (B) in *B38* flies with and without *FBti0019170*
634 (C) and in *IV52* flies with and without *FBti0019170*.

635

Figure 8. **Flies homozygous for the presence and for the absence of *FBti0019170* showed no differences in *kuz* expression.**

Normalized expression level relative to *Act5C* of *kuz* in nonstress and zinc stress conditions in male and female flies. Flies without *FBti0019170* insertion are represented in grey and flies with *FBti0019170* are represented in red. Error bars represent the SEM of three biological replicas.

643 **Table 1. Log-rank analysis results and odds-ratio of heavy metal stress experiments performed with adult flies.**
644

Compared Strains	**Sex**	**Replica 1**		**Replica 2**	
		P-value	Odds ratio	P-value	Odds ratio
kuz-overexpressing vs. kuz-wildtype (Fig. 2)	Male	<< 0.001	**2.37** (1.7-3.32)	<< 0.001	**3.18** (2.25-4.50)
	Female	<< 0.001	**2.19** (1.57-3.057)	<< 0.001	**3** (2.12-4.24)
Outbred *FBti0019170(+) vs.* Outbred *FBti0019170(-)* (Fig. 4)	Male	<< 0.001	**2.32** (1.65-3.2395)	<< 0.001	**2** (1.44-2.78)
	Female	<< 0.001	**1.97** (1.42-2.74)	<< 0.001	**2.45** (1.75-3.43)
IV22 FBti0019170(+) vs. *B47 FBti0019170(-)* (Fig. 6A-6B)	Male	<< 0.001	**5.38** (3.6633-7.91)	<< 0.001	**2.85** (2.02-4.012)
	Female	<< 0.001	**4** (2.78-5.75)	<< 0.001	**3.23** (2.27-4.58)
IV145 FBti0019170(+) vs *B47 FBti0019170(-)* (Fig. 6C)	Male	<< 0.001	**33.22 (13.11-84.22)**	-	-
	Female	<< 0.001	**2.45 (1.64-3.67)**	-	-
B45 FBti0019170(+) vs *B47 FBti0019170(-)* (Fig. 6D)	Male	0.005	**1.76 (1.18-2.61)**	-	-
	Female	0.033	**2.11 (1.42-3.15)**	-	-

645
646

647 **Table 2. Two-way ANOVA analyses of egg to adult viability experiments.**

648

Strains analyzed	Two-way ANOVA					
	Experimental condition		Insertion genotype		Interaction between experimental condition & insertion genotype	
	P-value	Effect size	P-value	Effect size	P-value	Effect size
kuz-overexpressing vs kuz-wildtype (Fig. 3)	<<0.001	0.649	0.077	-	0.003	0.147
Outbred *FBti0019170 (+) vs outbred FBti0019170 (-)* (Fig. 5)	<<0.001	0.688	<<0.001	0.299	0.489	-
B47 FBti0019170 (-) vs IV22 FBti0019170 (+) (Fig. 7A)	<<0.001	0.682	0.001	0.115	<<0.001	0.138
B38 FBti0019170 (-) vs B38 FBti0019170 (+) (Fig. 7B)	<<0.001	0.750	0.426	-	<<0.001	0.146
IV52 FBti0019170 (-) vs IV52 FBti0019170 (+) (Fig. 7C)	<<0.001	0.756	0.395	-	0.399	-

649

Figure 1

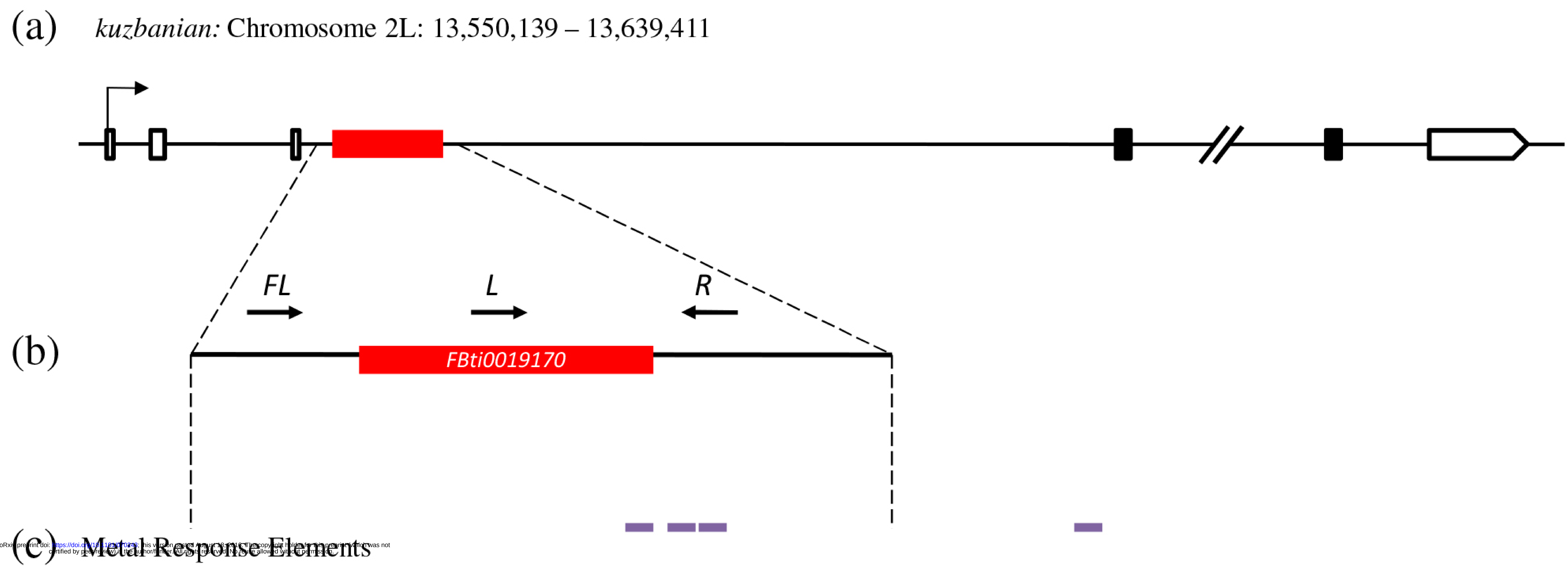

Figure 2

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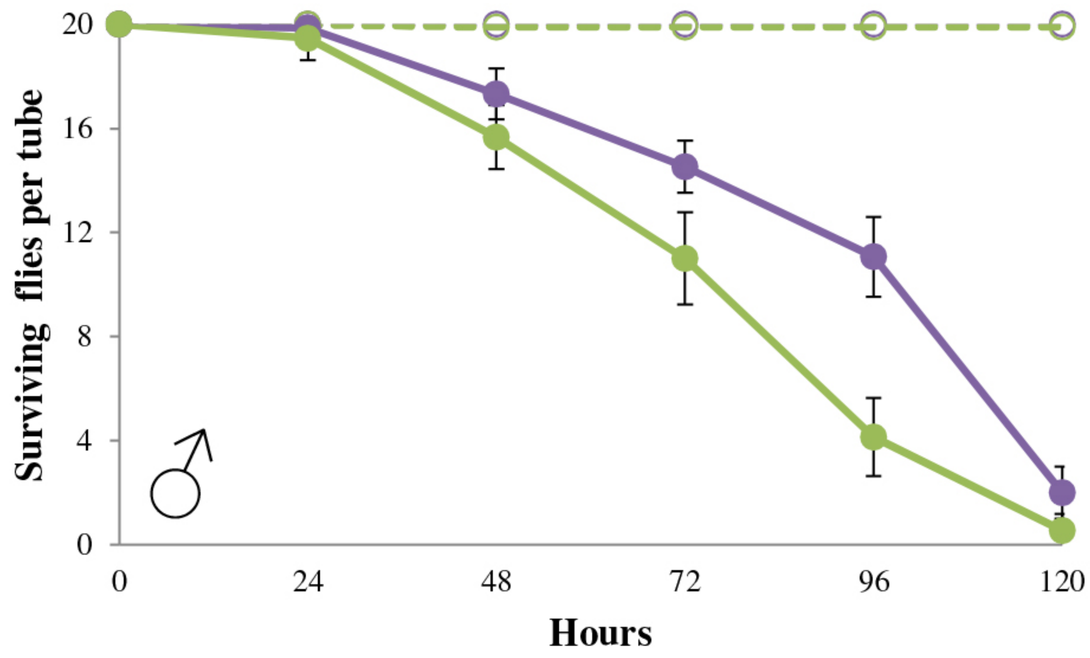

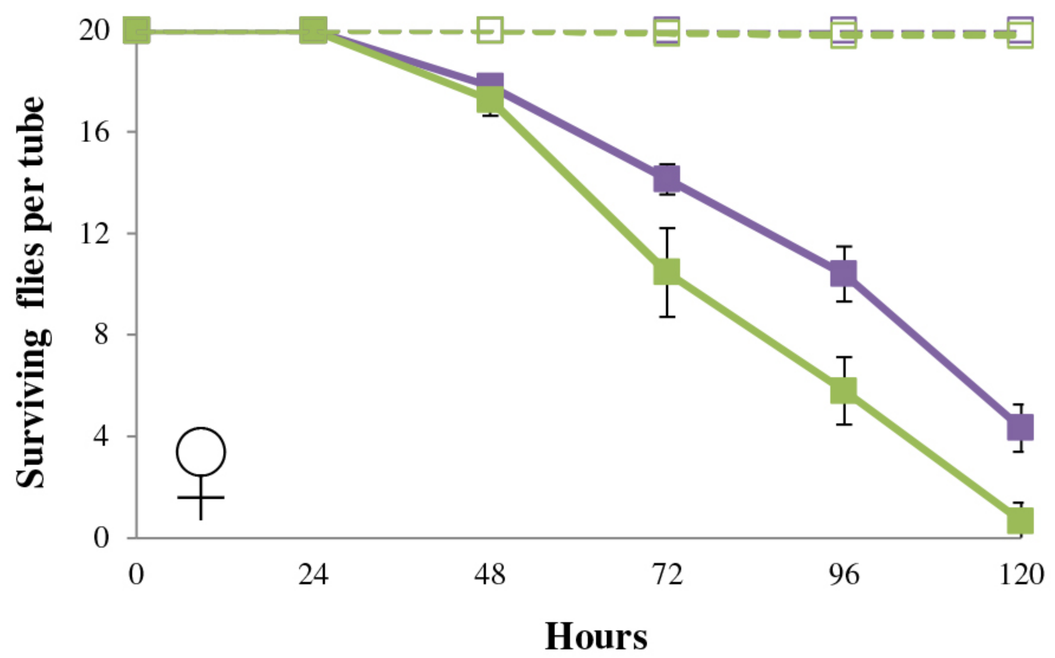

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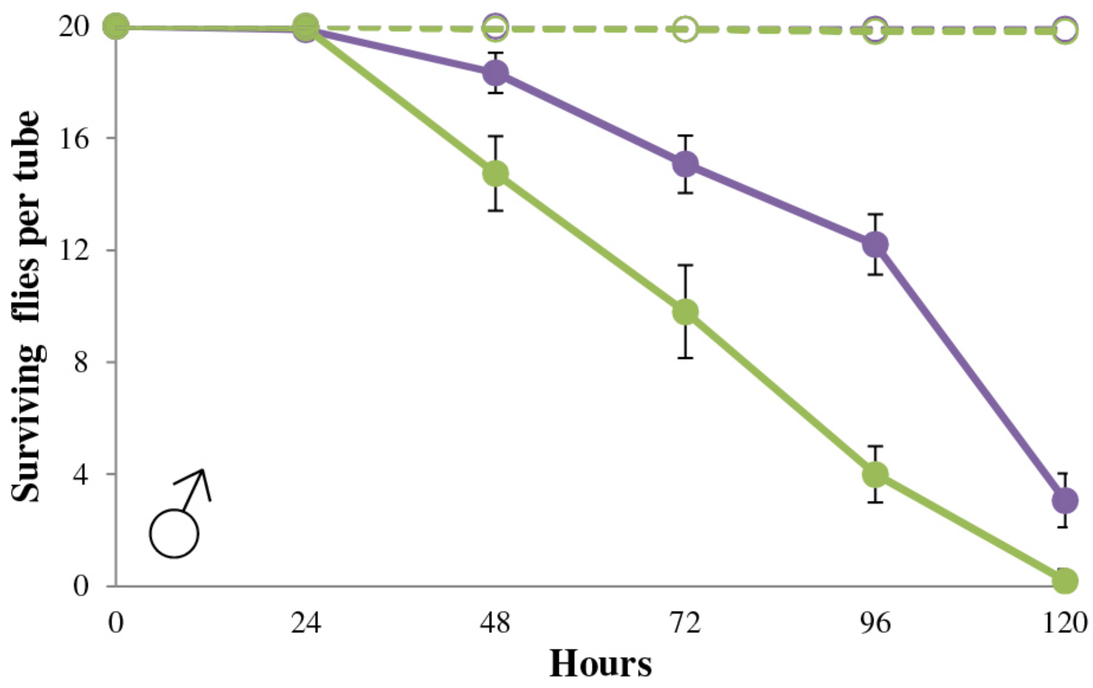
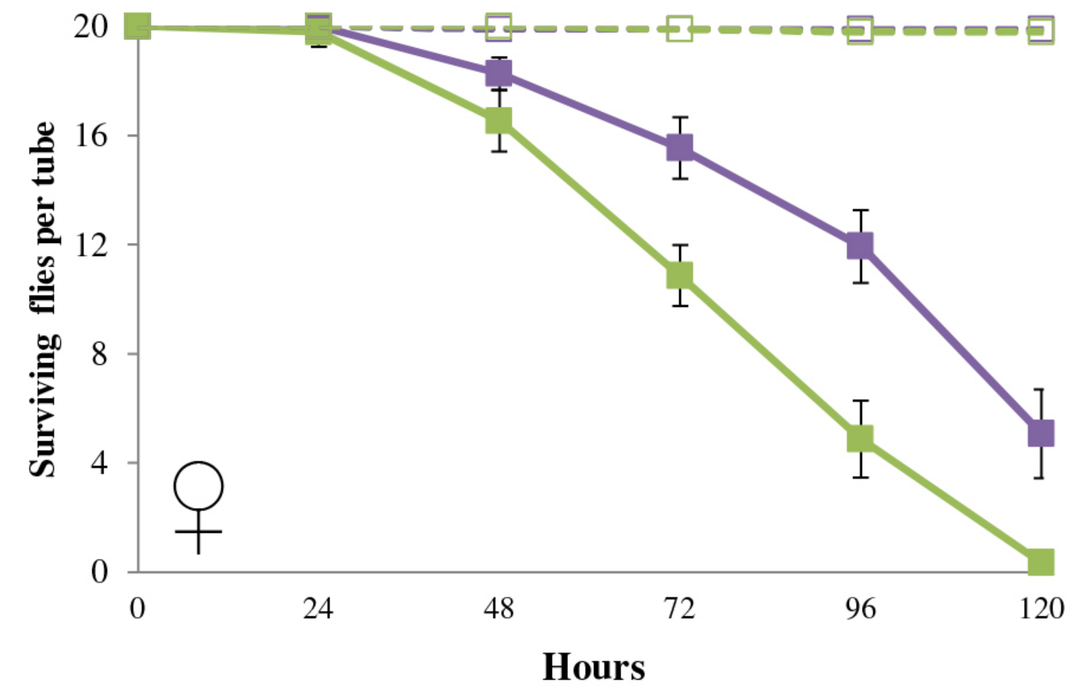




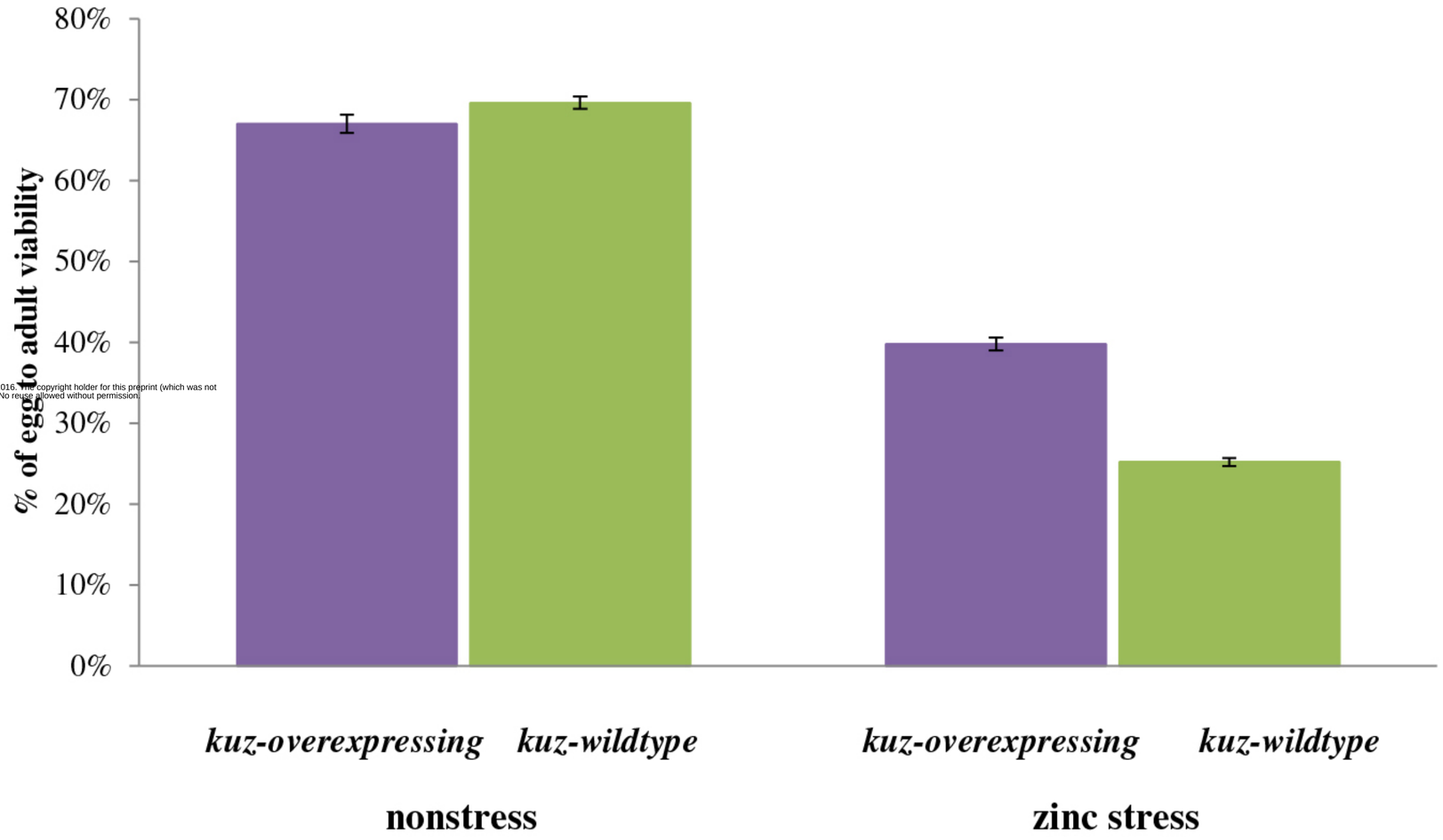
kuz-overexpressing

kuz-overexpressing zinc stress

kuz-wildtype nonstress

kuz-wildtype zinc stress

Figure 3



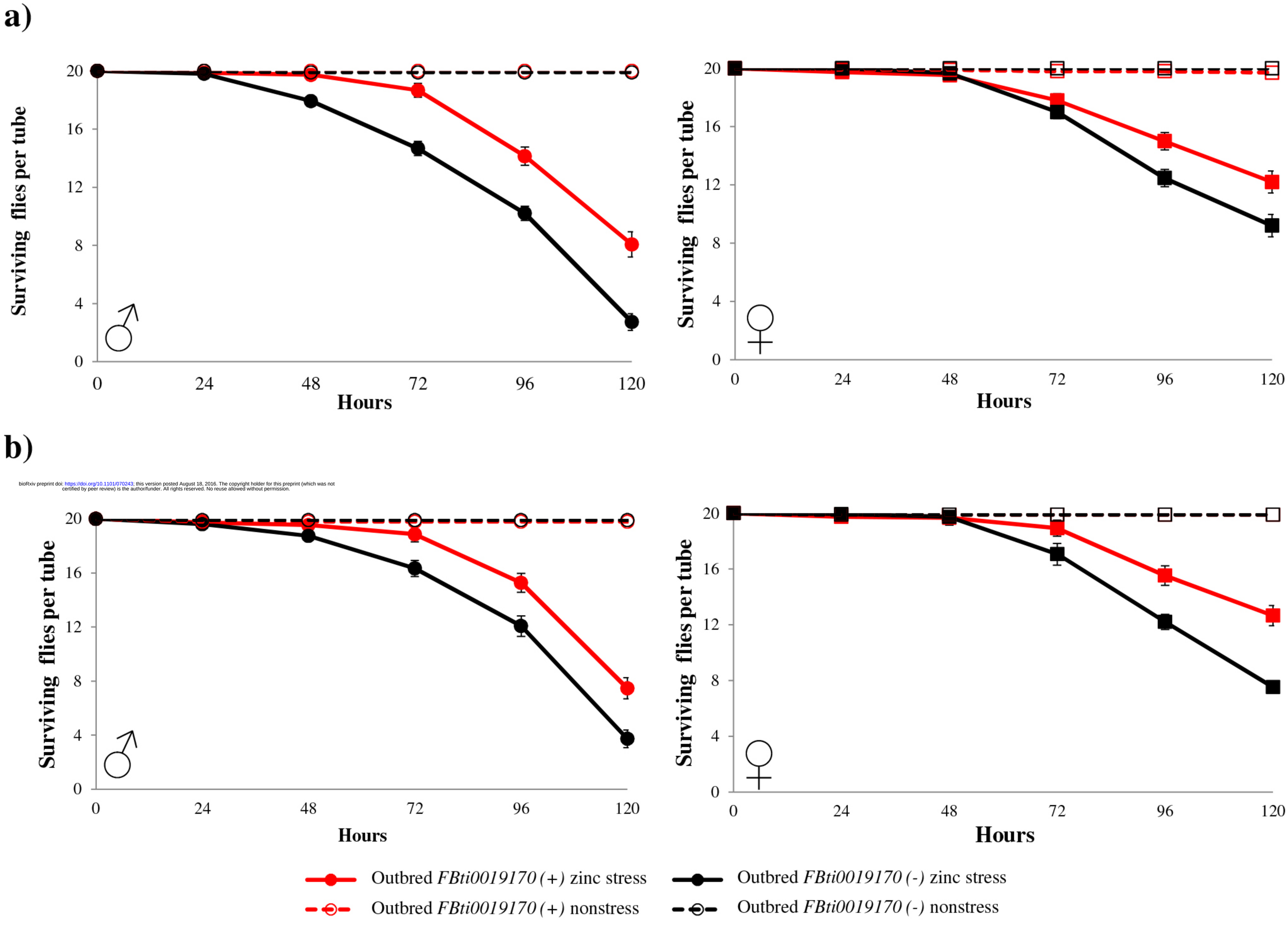


Figure 5

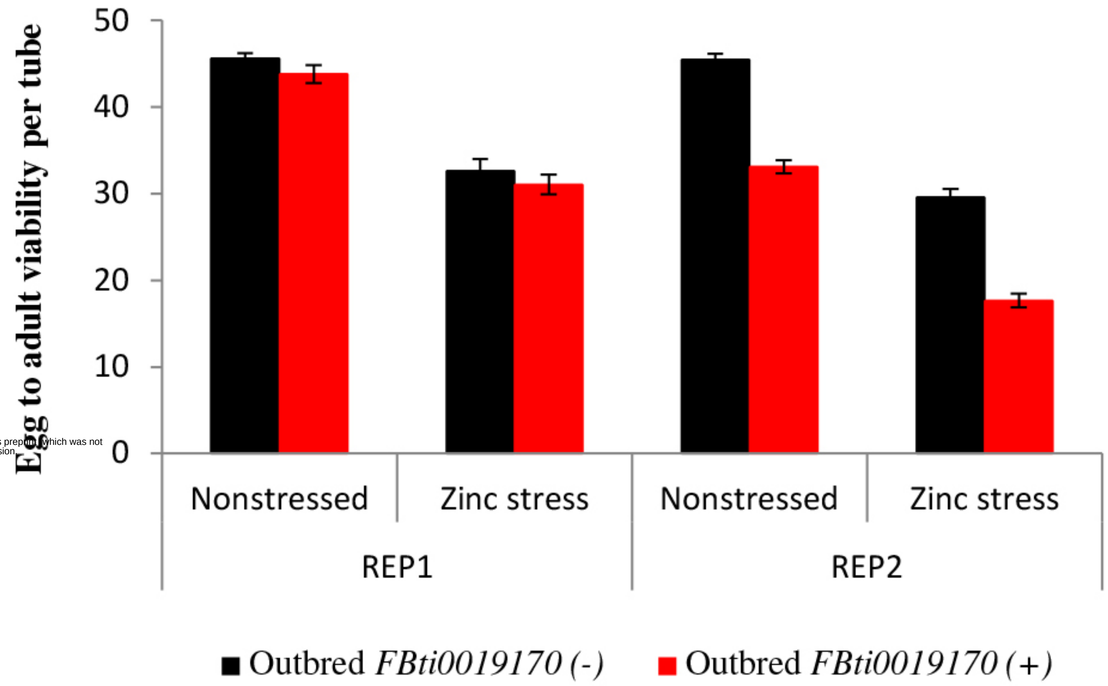
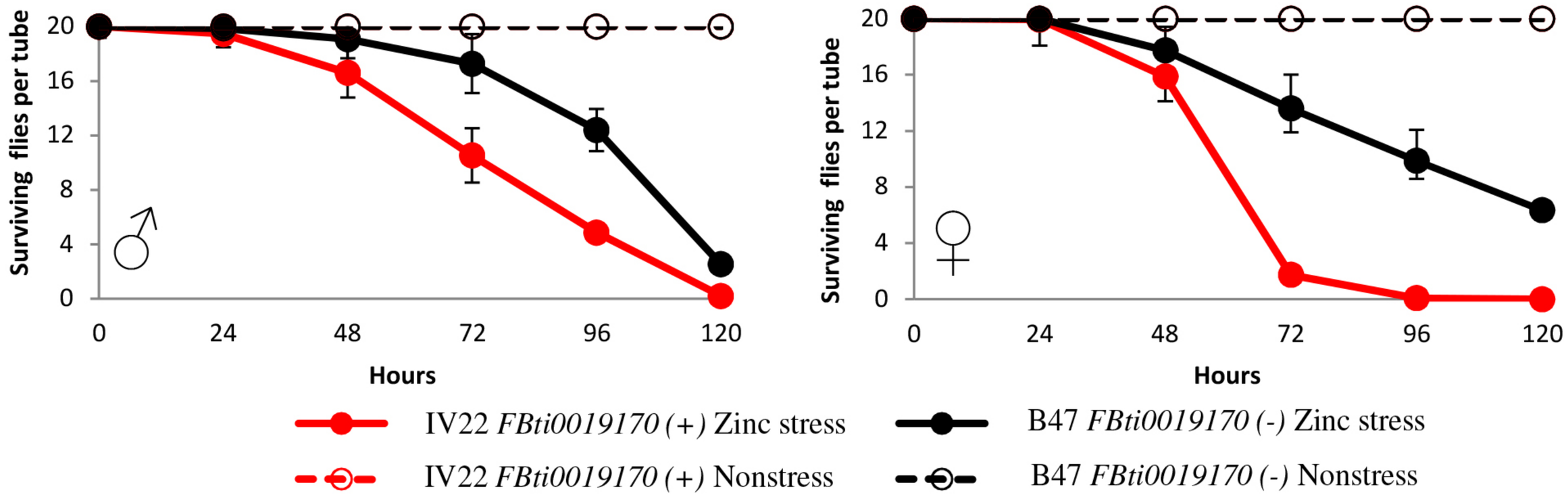


Figure 6

a)



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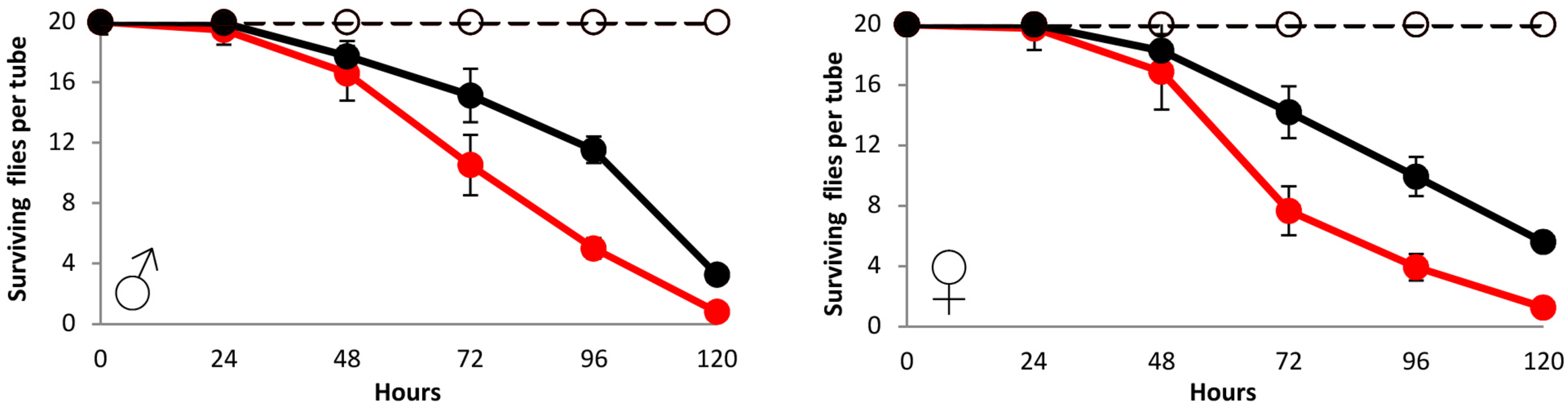

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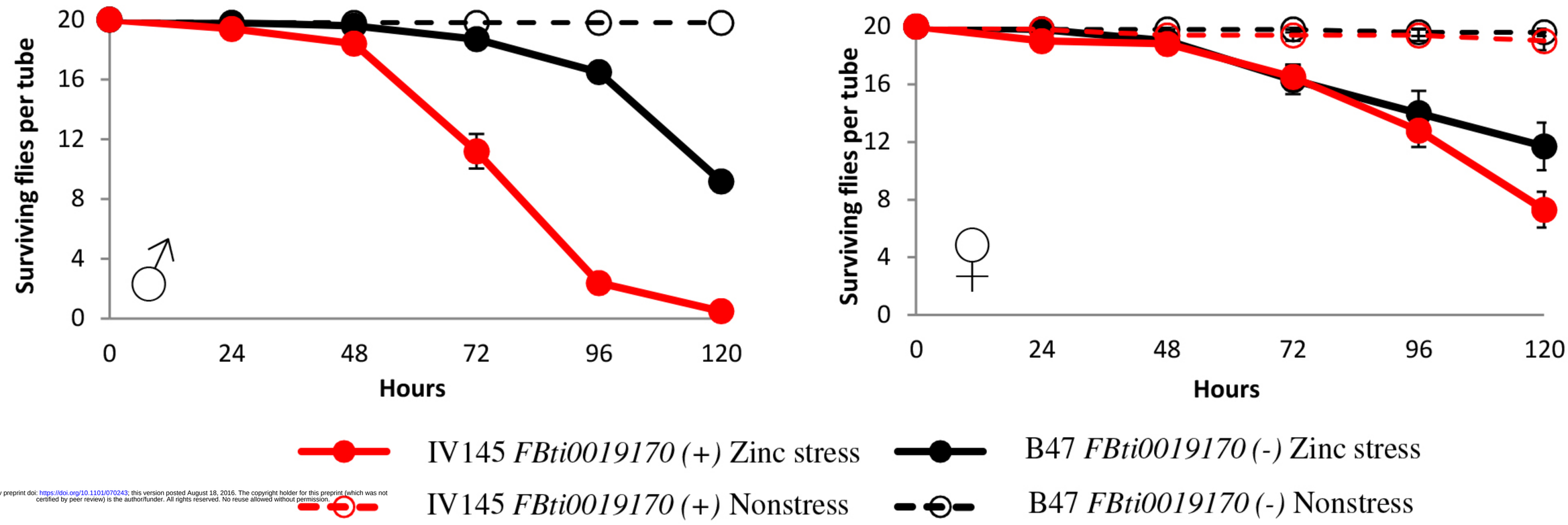
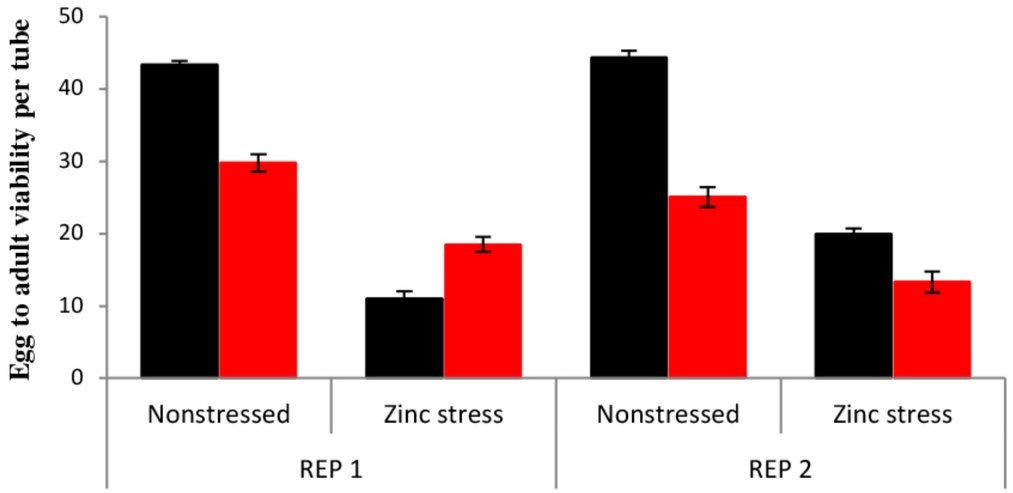


d)

a)



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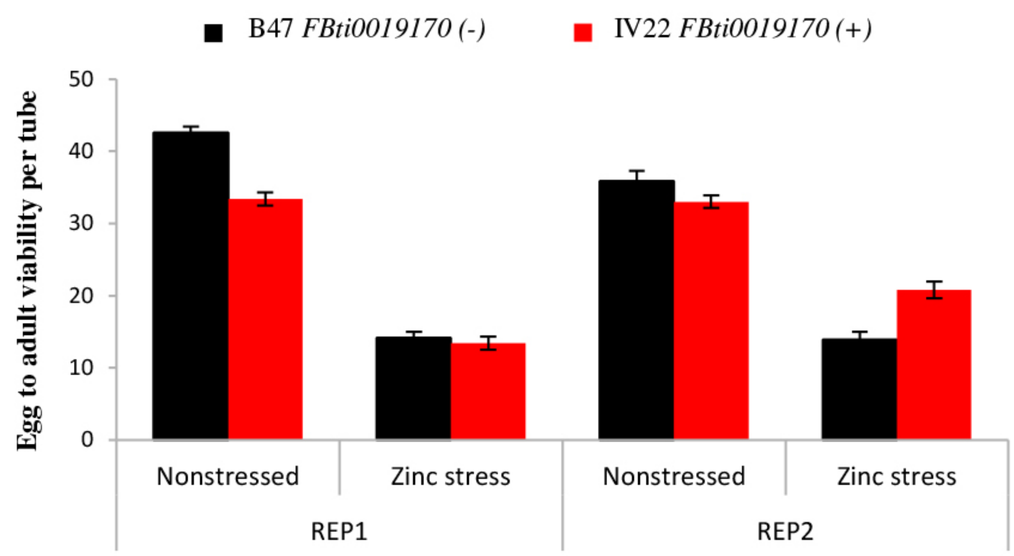

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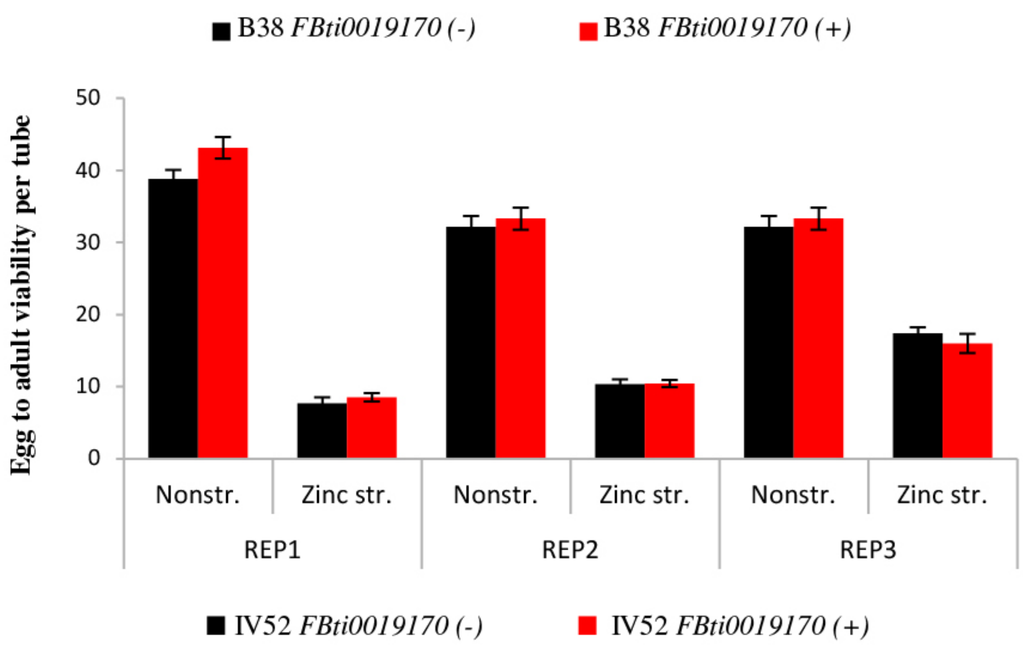

Figure 8