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Eric Le Bourg. A mild heat stress increases resistance to heat of dFOXO *Drosophila melanogaster* mutants but less in wild-type flies. *Biogerontology*, 2021, 22 (2), pp.237-251. <10.1007/s10522-021-09914-1>. <hal-03174536>

HAL Id: hal-03174536

<https://hal.science/hal-03174536v1>

Submitted on 19 Mar 2021

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A mild heat stress increases resistance to heat of *dFOXO Drosophila melanogaster* mutants but less in wild-type flies

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Electronic supplementary material

The online version of this article (doi: currently unknown) contains supplementary material, which is available to authorised users.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Abstract

While severe stresses have deleterious effects, mild stresses can have beneficial effects called hormetic effects. This study observed survival time at 37.5 °C and at 13-16 days of age of wild-type *Drosophila melanogaster* flies and *dFOXO* mutants, after they were subjected to 5 or 10 minutes daily at 37.5 °C for 5 days starting at 5 days of age. This mild stress increased survival time of the mutants, this effect being nearly not observed in wild-type flies. Previous studies showed that another mild stress, the cold, can increase survival time to heat of wild-type flies, but not of *dFOXO* mutants, while hypergravity increased survival time of mutants but not of wild-type flies. Therefore, three mild stresses, cold, hypergravity, and heat can increase resistance to heat but the pathways mediating this effect are seemingly different, as cold does not increase resistance in *dFOXO* mutants but increases it in wild-type flies, while hypergravity and heat have opposite effects. It appears that *dFOXO* may be needed or not to observe hormetic effects.

Key-words

Hormesis — Mild stress — Heat stress — *dFOXO* mutants — *Drosophila melanogaster*

Introduction

It is now well known that mild stresses can have beneficial effects, called hormetic effects (Mattson and Calabrese 2010), by inducing adaptive responses increasing the resistance to severe stress. This can be observed in flies, nematodes, mammals, including human beings (reviews in e.g. Le Bourg 2009; Rattan and Le Bourg 2014; Rattan and Kyriazis 2019), but the mechanisms explaining these effects are still elusive. Studies in *Drosophila melanogaster* showed that two antioxidant enzymes (superoxide dismutase and catalase) are probably not involved (Le Bourg and Fournier 2004), that heat-shock proteins (HSP70) could explain, at least partly, the better resistance to heat (Le Bourg et al. 2002; Kristensen et al. 2003; Sørensen et al. 2007), and that the NF- κ B-like transcription factor DIF (dorsal-related immunity factor) could explain the better resistance to fungi and heat, but not to cold (Le Bourg et al. 2012). These studies pretreated flies with different mild stresses (heat, cold, and hypergravity, i.e. gravity levels higher than 1g, the Earth gravity level: 3 or 5 g) before observing resistance to a severe stress.

The Forkhead box class O (FOXO) transcription factor, a downstream effector of the insulin/insulin-like growth factor-1 pathway, is involved in regulation of metabolism and resistance to stress in *D. melanogaster* (review in Puig and Mattila 2011). This transcription factor remains in the cytoplasm when nutrients are abundant but translocates to the nucleus in starvation conditions

where it directs the expression of genes involved in resistance to various stresses. In the laboratory, *dFOXO* mutant flies are viable (e.g. Jünger et al. 2003), which could be an argument for a minor role of FOXO, but flies live in the wild where fasting episodes can be frequent. Increasing the responses to various stresses can then be crucial, because starved organisms can be frailer and starvation in the wild can occur concomitantly with other stresses. For instance, *dFOXO* modulates heat-shock proteins, at play against heat shock (Donovan and Marr 2016), but also induces the translocation of the innate immunity transcription factor Relish involved in resistance to hypoxia (Barretto et al. 2020), or the expression of anti-microbial genes involved in resistance to bacteria (Fink et al. 2016).

The role in mediating the positive effects of mild stress on resistance to severe stress has also been studied. Fasting, a mild stress increasing resistance of wild-type flies to a severe cold stress (Le Bourg 2013), could also slightly increase that of *dFOXO* mutants (Le Bourg and Massou 2015), but a mild irradiation did not increase tolerance of the same mutants to a strong irradiation, contrarily to what was observed in wild-type flies (Moskalev et al. 2011). A mild cold stress increased survival time at 37 °C of wild-type flies, but not that of two *dFOXO* mutants (Polesello and Le Bourg 2017). The heat stress strongly increased *dFOXO* nuclear translocation, but to a less extent in cold-pretreated wild-type males, the cold pretreatment alone having nearly no effect. Because cold-pretreated wild-type males survived heat longer and had a lower *dFOXO* translocation after a heat stress than not-pretreated flies, it seems that, although *dFOXO* is required to resist heat, other mechanisms partly substitute to *dFOXO* translocation in cold-pretreated flies. After this study, the survival time at 37 °C of wild-type flies and *dFOXO* mutants was observed after they lived or not for two weeks in hypergravity (3 or 5 g). Hypergravity increased survival time of the mutants, this effect being less or not observed in wild-type flies (Le Bourg and Polesello 2019). The heat stress increased *dFOXO* translocation similarly in all gravity groups in a wild-type strain, and hypergravity decreased *dFOXO* translocation similarly in heat-stressed or not heat-stressed males, no clear effect of hypergravity being observed in females. Because hypergravity increased resistance to heat in *dFOXO* mutants and the translocation was not tightly dependent on the gravity level, one can conclude that *dFOXO* does not mediate the effect of hypergravity on resistance to heat. Therefore, two mild stresses, cold and hypergravity, can increase resistance to heat but the pathways mediating this effect are seemingly different, as cold does not increase resistance in *dFOXO* mutants while hypergravity increases it.

A puzzling result is that the positive effect of hypergravity on resistance to heat was more important in mutants than in wild-type flies, which allows to suspect that *dFOXO* could be in some cases an obstacle to the existence of hormetic effects or, at least, that it does not mediate hormetic

effects in some cases. One can thus wonder what is the exact role of dFOXO in mediating resistance to severe stress after a mild stress and it is of interest to make use of a third mild stress known to increase resistance to heat and lifespan, as mild cold stress and hypergravity do (review in Le Bourg 2009). Short heat stresses have been shown to very slightly increase survival time at 37 °C and lifespan (Le Bourg et al. 2001) in the same Meyzieu wild-type strain as the one used by Polesello and Le Bourg (2017) and Le Bourg and Polesello (2019). The window of these effects was narrow, as only 5 and 10 min heat shocks had positive effects, while longer ones were detrimental or neutral. Because the positive effects were not impressive, this mild stress was no longer used in subsequent studies, mild cold stress and hypergravity having more positive effects. However, it could be wondered whether, as it is the case for hypergravity, *dFOXO* mutants would display more positive effects than wild-type flies. Indeed, it could be that only weak effects of mild heat shocks on resistance to a severe heat stress are observed in wild-type flies because dFOXO can, in some cases, prevent the occurrence of hormetic effects. Therefore, knowing whether dFOXO has similar effects when different mild stresses are used could allow to conclude whether dFOXO can be an important player in inducing hormetic effects. In addition to survival time at 37 °C, we also observed lifespan of flies subjected or not to the mild heat stress.

The mutants used in this article have been previously described. The loss-of-function mutants *dFOXO*²¹ and *dFOXO*²⁵ are viable, have slightly reduced wings, and a higher sensitivity to hydrogen peroxide, but not to starvation, bacterial infection, heat shock, or heavy-metal stresses (Jünger et al. 2003). *dFOXO*⁴⁹⁴ mutants (Slack et al. 2011) were also reported to have shorter wings and a slightly lower body size, but no higher sensitivity to the oxidant paraquat. *dFOXO*⁴⁹⁴ mutants have also a reduced viability when compared to the previous mutants (see the supplemental material in Polesello and Le Bourg 2017, Le Bourg and Polesello 2019, and in the present article).

Material and methods

Strains were maintained by mass-mating in bottles. Flies were fed on a medium (agar, sugar, corn meal and killed yeast) containing a mould inhibitor (para-hydroxymethyl-benzoic acid) and enriched with live yeast at the surface of the medium. At emergence (see below the developmental conditions of each strain), flies of the appropriate genotypes were transferred under ether anaesthesia in groups of 15 flies of the same sex to 20 ml polystyrene vials. Flies, transferred to new vials twice a week, spent their life in an incubator: the temperature was 25 ± 0.5 °C and light was on from 07.00 to 19.00 h.

Meyzieu, Castanet, w¹¹¹⁸, and dFOXO⁴⁹⁴ flies

The wild-type strains Meyzieu, caught at the end of the 1970s near the city of Lyon (France),

and Castanet, caught in 1994 near the city of Toulouse (France) (Draye et al. 1994), the white-eye inbred wild-type strain w^{1118} (Hazelrigg et al. 1984), and the white-eye null mutant $dFOXO^{A94}$ (Slack et al. 2011) were used in this study. In order to obtain the parents of the experimental flies, flies laid eggs for one night in a bottle. About 50 pairs emerging from this bottle 9–10 days after egg-laying were transferred to bottles (ca 25 pairs in a bottle): these flies are the parents of the experimental flies. Experimental flies of these strains were obtained as follows: eggs laid by ca 5 day-old parents during one night on a Petri dish containing the medium coloured with charcoal and a drop of live yeast were transferred by batches of 25 into 80 ml glass vials. Viability and sex-ratio data of the three strains are reported in the Supplemental Material section (Table S1).

dFOXO 21/25 flies

The loss-of-function mutants $dFOXO^{21}$ ($y w; (sp/ CyO); dFoxo(rev21)/TM6B Tb Hu$) and $dFOXO^{25}$ ($y w; +; FRT82 dFoxo(25)/TM6B Tb Hu$) (Jünger et al. 2003) have no detectable dFOXO protein (Giannakou et al. 2008; Slack et al. 2011) while their heterozygotes have each 65 % protein levels when compared to wild-type flies (Giannakou et al. 2008). In order to obtain the parents of the experimental flies, $dFOXO^{21}$ and $dFOXO^{25}$ flies were allowed to lay eggs for one night in separate bottles. $dFOXO^{25}$ heterozygote virgin females and $dFOXO^{21}$ heterozygote males emerging 9–10 days after egg-laying from these bottles were mixed in bottles (up to 25 pairs in a bottle): these flies are the parents of the experimental flies. Experimental flies were obtained as follows: eggs laid by ca 5 day-old parents during one night on a Petri dish containing the medium coloured with charcoal and a drop of live yeast were transferred by batches of 50 into 80 ml glass vials. Pupae were sorted ($dFOXO^{25}$ and $dFOXO^{21}$ heterozygotes (25/+ and 21/+) are tubby: *Tb*) and, at emergence (duration of preimaginal development: 9–10 days), virgin $dFOXO^{21}/dFOXO^{25}$ transheterozygotes (21/25), on one hand, and a mix of virgin 21/+ and 25/+ flies (+/+ eggs are lethal due to the balancer chromosome), on the other hand, were transferred into 20 ml polystyrene vials. Viability and sex-ratio data are reported in the Supplemental Material section (Table S2).

Crosses between 25/+ and dFOXO^{A94} flies

25/+ flies were crossed with $dFOXO^{A94}$ ones to complete the results obtained with the 21/25 and $dFOXO^{A94}$ flies. Transheterozygotes were $dFOXO^{A94}/25$ and heterozygotes were $dFOXO^{A94}/+$. Offspring of the cross between $dFOXO^{A94}$ dams and 25/+ sires had a ca 30 % viability, while offspring of the reciprocal cross had a ca 65 % viability (see Table S3 in Le Bourg and Polesello 2019). Therefore, dams used in this article were of the 25/+ genotype. In order to obtain the parents of the experimental flies, $dFOXO^{A94}$ and $dFOXO^{25}$ flies were allowed to lay eggs for one night in separate bottles. 25/+ virgin females and $dFOXO^{A94}$ males emerging 9–10 days after egg-laying from these bottles were mixed in bottles (up to 25 pairs in a bottle): these flies are the parents of the

experimental flies. Experimental flies were obtained as follows: eggs laid by ca 5 day-old parents during one night on a Petri dish containing the medium coloured with charcoal and a drop of live yeast were transferred by batches of 25 into 80 ml glass vials. Viability and sex-ratio data are reported in the Supplemental Material section (Table S3).

Crosses between 21/+ and $dFOXO^{A94}$ flies

Finally, 21/+ flies were crossed with $dFOXO^{A94}$ ones to complete the results obtained with the previous crosses. Experimental flies were obtained using the same procedure as that used for the crosses between 25/+ and $dFOXO^{A94}$ flies: 21/+ females were crossed with $dFOXO^{A94}$ males. Transheterozygotes were $dFOXO^{A94}/21$ and heterozygotes were $dFOXO^{A94}/+$. Viability and sex-ratio data are reported in the Supplemental Material section (Table S4).

Heat pretreatment

From 5 to 9 days of age, in early morning, flies were transferred into empty polystyrene vials (27 mm diameter and 64 mm length: 28 ml), the plug containing absorbent cotton with 65 ml of distilled water to prevent desiccation. These vials were immediately put into a water-bath set at 37.5 °C for 5 or 10 min. A last group of flies was transferred into the same vials for 7 minutes and 30 seconds and kept in the incubator at 25 °C, and thus these flies were not subjected to heat. After that, flies were transferred back to their rearing vials.

Resistance to heat

The survival time in a water-bath set at 37.5 °C was observed at 13, 14, 15, and 16 days of age, thus ca one week after the last heat pretreatment. In each experiment, 48 flies were observed in each sex, genotype, and heat pretreatment group (n = 288 for each genotype, but see below). However, it was not possible to obtain the very same number of flies for each age. Flies were transferred in early morning, just before the heat shock, in groups of two flies into empty polystyrene vials (27 mm diameter and 64 mm length: 28 ml). These vials were put into a water-bath set at 37.5 °C, the plug containing absorbent cotton with 65 ml of distilled water to prevent desiccation (18 vials in the water-bath). Vials were observed with a headset magnifier every 5 min: flies totally immobile (body, legs, wings, head, proboscis) during 6 successive records were considered to be dead. The experimenter could identify the gender and genotype when observing flies but was blind to the heat pretreatment group.

For the Meyzieu strain, flies of the 35/2019 and 36/2019 groups were observed; for the $dFOXO^{A94}$ strain, flies of the 39/2019, 40/2019 and 41/2019 groups; for the cross between $dFOXO^{A94}$ and 25/+ flies, flies of the 44/2019, 45/2019, 46/2019, 47/2019, and 48/2019 groups; for the w^{1118} strain, flies of the 01/2020 and 02/2020 groups; for the 21/25 and the mix of 21/+ and 25/+ flies, flies of the 04/2020, 06/2020, 07/2020, and 09/2020 groups (because of the first 2020 French

lockdown during the coronavirus outbreak only 42 flies, and not 48 ones, were observed in each sex, genotype, and heat pretreatment group, $n = 252$ for each genotype). For the Castanet strain, flies of the 22/2020, 23/2020 and 24/2020 groups were observed; for the cross between $dFOXO^{A94}$ and 21/+ flies, flies of the 45/2020, 46/2020, 47/2020, and 48/2020 groups.

For each experiment, survival times were analysed with an analysis of variance testing the effect of sex, heat pretreatment, genotype (if it was a factor), and all interactions. The age factor (13, 14, 15 or 16 days of age) was not included in the design.

Lifespan

Vials of 15 flies were observed daily from the first day of adult life up to the death of the last fly in each sex and heat pretreatment group. For the Meyzieu strain, 6 vials of the 34/2019 group were observed in each group (most flies of one vial of females with a 10 min heat pretreatment were lost at 3 days of age); for the $dFOXO^{A94}$ strain, 2-3 vials of the 42/2019 and 43/2019 groups; for the $dFOXO^{A94}/25$ and $dFOXO^{A94}/+$ genotypes, 2-3 vials of the 44/2019 group; for the w^{1118} strain, 6-7 vials of the 01/2020 group; for the 21/25 and the mix of 21/+ and 25/+ flies, ca 8 vials for the mix of 21/+ and 25/+ flies and 2-3 vials for 21/25 ones of the 07/2020 and 09/2020 groups; for the Castanet strain, 6 vials of the 35/2020 group; for the $dFOXO^{A94}/21$ and $dFOXO^{A94}/+$ genotypes, 2-3 vials of the 37/2020 group. For each experiment, the effects of sex, heat pretreatment, genotype (if it was a factor) and all interaction(s) on lifespan were analysed with an analysis of variance.

Results

Wild-type flies: Meyzieu, w^{1118} , and Castanet strains

Females of the Meyzieu wild-type strain better resisted than males (Fig. 1A and S1A, $F(1, 282) = 24.11$, $p < 0.0001$, mean \pm SEM: 133.72 ± 4.26 vs 109.93 ± 2.27 min). The effect of the heat pretreatment and its interaction with sex were not significant (Fs close to 1), even if there was a very slight tendency for a better survival in pretreated groups. Thus, the heat pretreatment did not increase survival time to heat in both sexes of this wild-type strain. Females lived longer than males (Fig. S3AB, $F(1, 478) = 84.88$, $p < 0.0001$, 56.70 ± 0.50 vs 48.62 ± 0.72 days). The pretreatment had no effect (F close to 1), as well as its interaction with sex (F close to 2), even if lifespan slightly increased in females when the time at 37 °C increased.

Females of the w^{1118} inbred strain better resisted than males (Fig. 1B and S1B, $F(1, 282) = 204.43$, $p < 0.0001$, means \pm SEM: 180.07 ± 2.45 vs 134.62 ± 2.03 min). The effect of the pretreatment and its interaction with sex were not significant (Fs close to 1). Thus, the pretreatment did not increase survival time. Females lived longer than males (Fig. S3CD, $F(1, 549) = 41.81$, $p < 0.0001$, 64.28 ± 0.59 vs 57.92 ± 0.80 vs days). The pretreatment decreased lifespan ($F(2, 549) =$

12.21, $p < 0.0001$; control, 5 and 10 min groups, respectively: 64.39 ± 0.70 , 61.10 ± 0.99 , 58.68 ± 0.86 days), and the interaction with sex showed that this effect was mainly due to males ($F(2, 549) = 4.61$, $p = 0.0103$; control, 5 and 10 min males, respectively: 63.21 ± 1.08 , 57.06 ± 1.52 , 54.06 ± 1.32 days; control, 5 and 10 min females, respectively: 65.39 ± 0.90 , 64.35 ± 1.21 , 63.10 ± 0.92 days). Thus, the pretreatment decreased lifespan, mainly in males.

Females of the Castanet wild-type strain better resisted than males (Fig. 1C and S1C, $F(1, 282) = 59.48$, $p < 0.0001$, mean \pm SEM: 153.96 ± 2.98 vs 127.54 ± 1.80 min). The effect of the pretreatment was not significant (F close to 1) but its interaction with sex ($F(2, 282) = 4.78$, $p = 0.0091$) showed that the pretreatment increased survival time in females (control, 5 and 10 min females, respectively: 143.13 ± 4.57 , 157.19 ± 5.66 , 161.56 ± 4.91 min) but not in males (control, 5 and 10 min males, respectively: 129.90 ± 2.84 , 130.31 ± 3.02 , 122.40 ± 3.38 min). Females lived slightly longer than males (Fig. S3EF, $F(1, 485) = 5.15$, $p = 0.0237$, 55.91 ± 0.58 vs 53.86 ± 0.68 days). The pretreatment and its interaction with sex had no effect on lifespan (F s close to 1).

dFOXO and control flies

Females of the *dFOXO^{A94}* strain better resisted than males (Fig. 2A and S2A, $F(1, 282) = 49.36$, $p < 0.0001$; 171.32 ± 5.07 vs 121.94 ± 5.07 min). The pretreatment increased survival time, mainly in the 10 min group ($F(2, 282) = 7.63$, $p = 0.0006$; control, 5 and 10 min groups, respectively: 132.34 ± 6.02 , 142.40 ± 6.51 , 165.16 ± 7.13 min). The interaction with sex was not significant (F close to 1). Thus, pretreated *dFOXO^{A94}* flies longer survived heat, and this effect was more important in the 10 min group. Males lived slightly longer than females (Fig. S4AB, $F(1, 483) = 5.44$, $p = 0.0201$, 28.27 ± 0.68 vs 25.87 ± 0.68 days). The pretreatment had no effect, as well as its interaction with sex (F s close to 1).

Females of the *dFOXO^{A94/25}* and *dFOXO^{A94/+}* genotypes better resisted than males (Fig. 2BC, Fig. S2BC, $F(1, 564) = 393.18$, $p < 0.0001$; 196.86 ± 2.38 vs 132.90 ± 2.34 min) and *dFOXO^{A94/+}* flies slightly better resisted than *dFOXO^{A94/25}* ones ($F(1, 564) = 10.78$, $p < 0.0011$; 170.17 ± 2.77 vs 159.58 ± 3.23 min). The pretreatment increased survival time, mainly in the 10 min group ($F(2, 564) = 10.40$, $p < 0.0001$; control, 5 and 10 min groups, respectively: 156.62 ± 3.30 , 163.54 ± 4.03 , 174.48 ± 3.63 min). The second-order interaction between sex, heat pretreatment and genotype showed that this effect was mainly due to females in *dFOXO^{A94/+}* flies and to males in *dFOXO^{A94/25}* ones ($F(2, 564) = 5.52$, $p = 0.0042$). The other interactions were not significant. Thus, a positive effect of the pretreatment was observed in *dFOXO^{A94/25}* and in *dFOXO^{A94/+}* flies, mainly in males and females respectively. Females lived longer than males (Fig. S4CDEF, $F(1, 377) = 366.04$, $p < 0.0001$, 51.52 ± 0.92 vs 30.77 ± 0.82 days) and *dFOXO^{A94/+}* flies lived longer than *dFOXO^{A94/25}* ones ($F(1, 377) = 132.59$, $p < 0.0001$, 46.74 ± 1.05 vs 34.03 ± 1.03 days), the significant interaction

between the sex and genotype factors ($F(1, 377) = 38.49$, $p < 0.0001$) showing that the sex effect was more important in *dFOXO^{Δ94/+}* flies (59.26 ± 0.87 vs 33.62 ± 0.79 days) than in *dFOXO^{Δ94/25}* ones (41.17 ± 0.98 vs 27.37 ± 1.46 days). The pretreatment had no effect (F close to 2) and the just significant genotype by pretreatment interaction ($F(2, 377) = 3.18$, $p = 0.0425$) showed that, among *dFOXO^{Δ94/25}* flies, those subjected to 10 min heat shocks lived shorter than those subjected to 5 min heat shocks and control flies, no such effect being observed in *dFOXO^{Δ94/+}* flies. The other interactions were not significant (F s close to 1). Thus, the pretreatment had no effect on lifespan in both genotypes and, if any, it was a deleterious effect.

For the 21/25 and the mix of 21/+ and 25/+ flies, females better resisted than males (Fig. 2DE, Fig. S2DE, $F(1, 492) = 83.54$, $p < 0.0001$; 158.61 ± 3.48 vs 118.41 ± 3.25 min). The 21/25 flies survived for a lower time than heterozygotes ($F(1, 492) = 83.21$, $p < 0.0001$; 118.45 ± 4.03 vs 158.57 ± 2.54 min). The effect of the pretreatment was not significant ($F(2, 492) = 2.49$), but there was a slight tendency for a better resistance in flies subjected to the 10 min heat shocks that was mainly due to 21/25 flies. All interactions were not significant (F s close or lower than 1). Thus, a positive effect of the pretreatment is not clearly observed in these *dFOXO* and control flies. Females lived longer than males (Fig. S4GHIJ, $F(1, 1010) = 55.66$, $p < 0.0001$, 41.29 ± 0.69 vs 35.17 ± 0.72 days) and the mix of 21/+ and 25/+ flies lived longer than 21/25 ones ($F(1, 1010) = 719.14$, $p < 0.0001$, 44.30 ± 0.44 vs 20.31 ± 0.84 days). The pretreatment had a very slight negative effect in the 10 min group ($F(2, 1010) = 3.85$, $p = 0.0216$; control, 5 and 10 min groups, respectively: 39.07 ± 0.90 , 39.24 ± 0.91 , 37.31 ± 0.83 days) and all interactions were not significant (F s close to 0 or 2). Thus, the pretreatment had no clear effect on lifespan in both genotypes and, if any, it was a very slight deleterious effect in the 10 min group.

Females of the *dFOXO^{Δ94/21}* and *dFOXO^{Δ94/+}* genotypes better resisted than males (Fig. 2FG, Fig. S2FG, $F(1, 564) = 287.02$, $p < 0.0001$; 189.83 ± 2.29 vs 135.43 ± 2.41 min) and *dFOXO^{Δ94/21}* flies slightly better resisted than *dFOXO^{Δ94/+}* ones ($F(1, 564) = 7.94$, $p = 0.0050$; 167.15 ± 3.24 vs 158.11 ± 2.36 min). The pretreatment increased survival time, mainly in the 10 min group ($F(2, 564) = 13.13$, $p < 0.0001$; control, 5 and 10 min groups, respectively: 156.28 ± 3.66 , 157.37 ± 3.48 , 174.25 ± 3.15 min). The sex by heat pretreatment interaction ($F(2, 564) = 3.46$, $p = 0.0321$) showed that the pretreatment effect was more important in males (control, 5 and 10 min males, respectively: 123.13 ± 3.88 , 132.76 ± 4.11 , 150.42 ± 4.08 min) than in females (control, 5 and 10 min females, respectively: 189.43 ± 3.94 , 181.98 ± 4.38 , 198.07 ± 3.37 min). The other interactions were not significant, even if the effect of the pretreatment was more important in *dFOXO^{Δ94/21}* flies. Thus, a positive effect of the pretreatment was observed in *dFOXO^{Δ94/21}* and in *dFOXO^{Δ94/+}* flies, mainly in males. Females lived longer than males (Fig. S4KLMN, $F(1, 374) = 187.81$, $p < 0.0001$, $54.59 \pm$

0.99 vs 36.39 ± 0.97 days) and *dFOXO*^{A94/+} flies lived longer than *dFOXO*^{A94/21} ones ($F(1, 374) = 36.69$, $p < 0.0001$, 51.19 ± 1.20 vs 41.71 ± 1.07 days). The pretreatment slightly decreased lifespan, mainly in the 10 min group ($F(2, 374) = 9.56$, $p < 0.0001$; control, 5 and 10 min groups, respectively: 48.21 ± 1.48 , 47.70 ± 1.37 , 42.84 ± 1.45 days). All interactions were not significant (Fs close to 1). Thus, the pretreatment slightly decreased lifespan in both genotypes.

Comparing dFOXO and wild-type flies

Table 1 summarises the results on survival time at 37 °C: it seems that pretreated *dFOXO* flies (*dFOXO*^{A94}, *dFOXO*^{A94/25}, *dFOXO*^{A94/21}) survived longer than not-pretreated ones, this effect being however not significant in 21/25 flies, and that no effect of the pretreatment was observed in control sibling and wild-type flies, at least in one sex (Meyzieu, *w*¹¹⁸, Castanet, *dFOXO*^{A94/+} (controls of *dFOXO*^{A94/25} and of *dFOXO*^{A94/21}), mix of 21/+ and 25/+). To clarify the effect of the *dFOXO* loss of function mutation, new ANOVAs were done, *dFOXO* and all control flies genotypes being analysed in separate ANOVAs (factors: sex, genotype, pretreatment and all interactions). In both ANOVAs (Fig. 3), females survived longer than males (all control flies: $F(1, 1656) = 672.24$, $p < 0.0001$; 171.75 ± 1.55 vs 130.19 ± 0.95 min; *dFOXO* flies: $F(1, 1092) = 286.79$, $p < 0.0001$; 175.74 ± 2.36 vs 122.08 ± 2.42 min). The pretreatment effect was significant in control flies, but this was a very slight effect ($F(2, 1656) = 4.38$ $p = 0.0126$, control, 5 and 10 min groups, respectively: 148.87 ± 1.71 , 149.72 ± 1.74 , 152.33 ± 1.95 min). The pretreatment effect was also significant in *dFOXO* flies, mainly in the 10 min group, to a larger extent than observed in control flies ($F(2, 1092) = 20.91$, $p < 0.0001$, control, 5 and 10 min groups, respectively: 138.80 ± 3.05 , 144.92 ± 3.32 , 163.00 ± 3.22 min). The sex by pretreatment interaction was not significant in control flies (F close to 1), while the pretreatment effect was more important in *dFOXO* males ($F(2, 1092) = 3.44$, $p = 0.0324$, control, 5 and 10 min females, respectively: 169.22 ± 3.70 , 174.06 ± 4.21 , 183.93 ± 4.26 min; control, 5 and 10 min males, respectively: 108.39 ± 3.70 , 115.78 ± 4.15 , 142.07 ± 4.34 min). The genotype effect was significant (control flies: $F(5, 1656) = 78.41$, $p < 0.0001$; *dFOXO* flies: $F(3, 1092) = 43.84$, $p < 0.0001$), as well as the interaction between sex and genotype in control flies ($F(5, 1656) = 19.30$, $p < 0.0001$), but not in *dFOXO* ones (F close to 1). The interaction of the pretreatment and genotype factors and the second-order interaction between sex, genotype and pretreatment (Fs < 1) were not significant in *dFOXO* flies. By contrast, the second-order interaction between sex, genotype and pretreatment was significant in control flies ($F(10, 1656) = 2.53$, $p = 0.0050$), but not of a large magnitude.

To sum up, the pretreatment, mainly the 10 min one, increased the survival time at 37 °C of *dFOXO* flies, this effect being nearly not observed in control flies. Similar results showing an effect of the pretreatment in *dFOXO* flies and nearly no one in control flies are observed if one computes

an ANOVA with all flies, the genotype being considered as a random factor nested in the factor contrasting *dFOXO* flies vs all control ones (Table S5).

Regarding lifespan, new ANOVAs were also done, *dFOXO* and all control flies being analysed in separate ANOVAs (factors: sex, genotype, pretreatment and all interactions). Genotype, sex, and their interaction had significant effects in both *dFOXO* and control flies (Fs between 38.93 and 576.49, all p-values < 0.0001), showing the usual longer lifespan of females and the effects of the different genotypes. The pretreatment very slightly decreased lifespan of *dFOXO* flies (F(2, 1091) = 4.43, p = 0.0121; control, 5 and 10 min groups, respectively: 29.76 ± 0.77 , 29.68 ± 0.83 , 28.62 ± 0.68 days) and the interaction between genotype and pretreatment was explained by some erratic and minor differences with no trend (F(6, 1091) = 3.22, p = 0.0039), the other interactions being not significant. The pretreatment also slightly decreased lifespan of control flies (F(2, 2665) = 16.15, p < 0.0001; control, 5 and 10 min groups, respectively: 53.17 ± 0.44 , 51.50 ± 0.45 , 50.95 ± 0.46 days) and all interactions involving the pretreatment factor were significant, with no clear trend (Fs between 2.26 and 4.35, all p-values between 0.0130 and 0.0002). Thus, the pretreatment had no positive effect in *dFOXO* and control flies, but rather a slightly negative effect, if any.

Discussion

Mild stress can increase the resistance of *D. melanogaster* flies to severe stress, such as lethal heat (see references in the introduction), and different mild stresses are not efficient to the same extent. Short heat stresses were shown to very slightly increase survival time at 37 °C and lifespan (Le Bourg et al. 2001), while hypergravity (e.g. Le Bourg and Minois 1997) and mild cold stress (e.g. Le Bourg 2007) had larger effects on both phenotypes in the same Meyzieu wild-type strain, which explains why mild heat stress was no longer used in our lab to study hormetic effects. However, when studying whether *dFOXO* could explain the occurrence of hormetic effects, the puzzling result was that a pretreatment with cold stress increased survival time at 37 °C of wild-type flies, but not that of two *dFOXO* mutants (Polesello and Le Bourg 2017), while hypergravity increased survival time of these mutants, this effect being less or not observed in wild-type flies (Le Bourg and Polesello 2019). These contrasted results made necessary to use a third mild stress increasing survival time at 37 °C, i.e. mild heat stress. Because mild heat stress was expected to only barely increase survival time at 37 °C of wild-type flies (Le Bourg et al. 2001), it could be wondered whether, as for hypergravity, it would be less able to increase the resistance to lethal heat of wild-type flies than of *dFOXO* mutants, and also whether lifespan of wild-type and mutant flies subjected to this mild heat stress could increase.

Like for hypergravity, the mild heat stress increases survival time at 37 °C of *dFOXO* mutants

and this effect is much lower, if any, in wild-type flies. Positive effects of mild heat stress on resistance to a severe heat stress have been shown in males but not in females (Sørensen et al. 2007), in both sexes (e.g. Khazaeli et al. 1997; Dahlgaard et al. 1998; Le Bourg et al. 2001), or in females, males being not tested (Hercus et al. 2003). On the whole, a mild heat stress can increase resistance to a severe heat stress, but this is not always observed.

A slight lifespan decrease is observed in pretreated wild-type flies and *dFOXO* mutants, by contrast to the very weak positive effect (+5 %) observed by Le Bourg et al. (2001) in Meyzieu flies exposed to 37 °C for 5 min daily during 5 days, as in the present experiment. Deleterious or no effects of mild heat stress (35 min at 35.5 °C at 4 and 7 days of age) on lifespan were also observed in both sexes of nearly all recombinant-inbred lines (19/20) selected for high resistance to heat, while positive effects were observed in only one quarter of lines selected for a low resistance (males: 7/32, females 8/32) and deleterious effects in another quarter (8/32) (Defays et al. 2011). By contrast, Hercus et al. (2003) reported a 10 % positive effect on lifespan in females of a wild-type strain (3 hours at 34 °C at 3, 6, 9, and 12 days of age), but the same team (Sørensen et al. 2007) reported no effect in females of another, short-lived, strain pretreated at 3, 6 and 9 days of age and a ca 15 % positive effect in males. On the whole, it seems that mild heat stress has no clear effect on lifespan in various strains.

Therefore, while mild cold stress increases survival time at 37 °C of wild-type flies and decreases that of *dFOXO* mutants, hypergravity and mild heat stress increase this survival time in *dFOXO* mutants, a weaker effect, if any, being observed in wild-type flies (Fig. 4A). The main difference between these three pretreatments is that mild heat stress (this study) and hypergravity do not kill flies (e.g. Le Bourg et al. 2000), while the daily cold stresses can kill up to 70 % of females in 21/25 flies and in the mix of 21/+ and 25/+ ones (15 % of males also die), and even up to 90 % of *dFOXO^{A94}* flies (Polesello and Le Bourg 2017). Supplementary experiments in Le Bourg and Polesello (2019) confirmed this weak resistance of mutants to cold. However, the daily cold stresses also killed up to 30 % of Meyzieu females, but nearly no fly in the *w¹¹¹⁸* strain (Polesello and Le Bourg 2017). The positive effect of the cold stress on survival to heat was observed in both sexes of Meyzieu and *w¹¹¹⁸* flies and in males of the mix of 21/+ and 25/+ flies, the effect being slightly negative in females, and thus despite differences in resistance to cold among groups. However, even if 21/25 males better resisted than females to the cold pretreatment, this pretreatment decreased survival time at 37 °C in both sexes. On the whole, even if it could be hypothesised that mutant flies surviving to the daily cold stresses are frailer than not-pretreated ones, it seems that there is not a clear link between resistance to the daily cold pretreatments and survival time at 37 °C.

Thus, *dFOXO* mutants are unable to take advantage of a pretreatment with a cold stress to

survive longer at 37 °C, contrary to wild-type flies, but they longer survive at 37 °C if they have been pretreated with hypergravity or a mild heat stress, this positive effect being less or not observed in wild-type flies (Fig. 4A). A striking result is that not-pretreated *dFOXO* mutants survive shorter at 37 °C than not-pretreated wild-type flies, and for a similar duration if pretreated (Fig. 4B). It is the case when the pretreatment is hypergravity, provided its level is 5 g and not 3 g (Fig. 1 in Le Bourg and Polesello 2019), but also when a mild heat stress is used. Thus, being devoid of FOXO decreases resistance to heat but the mild stress allows to cope with this lack and pretreated *dFOXO* mutants eventually survive for the same duration as wild-type flies. It could be said that the mild stress has replaced FOXO as a means to help survive heat, but does it imply that mild stress is unable to have a significant positive effect in wild-type flies, because of a ceiling effect? As the pretreatment with cold can increase survival time at 37 °C of wild-type flies, it seems that a ceiling of resistance to heat has not been reached in these flies (Fig. 1 in Polesello and Le Bourg 2017).

One thus may conclude that the FOXO transcription factor is not necessarily mediating the hormetic effects of mild stress in flies, because *dFOXO* mutants can survive longer at 37 °C if they are subjected to hypergravity or mild heat pretreatments (see also fasting, Le Bourg and Massou 2015). However, FOXO is of some use when other mild stresses are used (cold: Polesello and Le Bourg 2017; irradiation: Moskalev et al. 2011), because the positive effect of mild stress is observed in wild-type flies but not in the mutants. Thus, the effect of *dFOXO* is variable, depending on the pretreatment preceding a severe stress, and it cannot simply be concluded that it mediates or not the hormetic effects in flies. *dFOXO* mutants have lower lifespan and resistance to heat than control ones, showing that the mutation increases frailty, but the same mutants display an increased resistance to heat after a pretreatment with a mild heat stress or hypergravity, while this effect is much lower in control flies, showing that *dFOXO* does not explain these hormetic effects.

The present study has confirmed that it could be possible to observe positive effects of a mild stress on survival to heat in *dFOXO* mutants. Thus, this result is not only observed with hypergravity but also with a mild heat stress, while no clear or weaker effects of these mild stresses are observed in wild-type flies. One could tentatively imagine, as an attempt to explain the existence of hormetic effects in *dFOXO* mutants and not in control flies subjected to some pretreatments, that the absence of *dFOXO* is a signal for implementing other pathways mediating hormetic effects that are not in use in the wild-type controls subjected to this pretreatment. In any case, this result deserves attention, as it implies that hormetic effects can be observed in mutants but not in their controls and, thus, studying mutants can bring to the fore phenotypes that can be hidden otherwise. In such conditions, one could imagine to search for other possible mechanisms of

hormetic effects by studying other mutants pretreated with a mild heat stress, because mild heat stress has only slight effects in wild-type strains: if these pretreated mutants would display a longer survival time at 37 °C, by contrast to their controls, it would mean that this genotype does not mediate this hormetic effect. The usual strategy, when studying mutants, is to look for impairments when compared to wild-type controls, and thus this is the opposite strategy: looking for improvements in mutants when only weak effects are expected in wild-type flies.

Another conclusion of these studies on the role of dFOXO in inducing hormetic effects (Polesello and Le Bourg 2017; Le Bourg and Polesello 2019; this study) is that despite various attempts to explain why a mild stress can increase resistance to severe stress or lifespan in flies, it is impossible to reach a firm conclusion. As indicated in the introduction of this article, superoxide dismutase and catalase are probably not involved (Le Bourg and Fournier 2004), heat-shock proteins (HSP70) could explain, at least partly, the better resistance to heat (Le Bourg et al. 2002; Kristensen et al. 2003; Sørensen et al. 2007), the NF- κ B-like transcription factor DIF could explain the better resistance to fungi and heat, but not to cold (Le Bourg et al. 2012), and dFOXO can explain the better resistance to heat if flies are pretreated with a mild cold stress, but not with hypergravity or a mild heat stress. However, heat-shock proteins and dFOXO are linked, because the transcription of heat-shock proteins in flies subjected to oxidative stress with the herbicide paraquat can be strongly lowered in dFOXO mutants (Donovan and Marr 2016).

Mechanisms of hormetic effects in flies appear to be multiple, being dependent on both the pretreatment and the severe stress, and there is thus no hope to discover THE mechanism explaining hormesis and thus the magic pill mimicking it, as some authors are trying to mimic the positive effects of calorie restriction often observed in rodents by looking for the molecule that could mimic its effects in humans (Madeo et al. 2019). This conclusion could seem to be over-pessimistic because studies in *Caenorhabditis elegans* have shown that DAF-16, the homologue of dFOXO, can be needed to observe hormetic effects in this nematode. A meta-analysis showed that DAF-16 was needed in the 7 articles studying the hormetic effects of chemicals (Sun et al. 2020; see also e.g. Schmeisser et al. 2013). DAF-16 was also required to observe hormetic effects of flavonoids or heat shock on lifespan, but it was not to observe increased thermotolerance after a heat shock or living in a H₂S atmosphere (review in Le Bourg 2009). However, while *daf-16* mutants like control worms show this increased thermotolerance one day after a mild heat stress, this effect is lost 4, 6, or 8 days after the mild heat stress, by contrast to what is observed in control worms (Dues et al. 2016). Therefore, like in flies, it appears that DAF-16 in worms may be needed or not to observe hormetic effects.

Acknowledgements

This work was partly supported by a grant from the “Centre de Biologie Intégrative, Université de Toulouse”. The *dFOXO*²¹ and *dFOXO*²⁵ flies were kindly provided by Hugo Stocker and Igor Vuillez (Institute of Molecular Systems Biology, ETH, Zurich, Switzerland) and the *dFOXO*¹⁹⁴ ones by Julien Colombani (Institut of Biology Valrose, University of Nice-Sophia-Antipolis, Nice, France).

Table 1: Summary of the effects of the heat pretreatment on survival time at 37 °C and on lifespan observed in each mutant or wild-type genotype. When a significant effect of the pretreatment on resistance to heat is observed, the percentage of increase for 5 and 10 min groups compared to the control group is indicated, a 0 meaning less than a 1 % variation. The results of the two sexes are reported when there is a significant interaction between the pretreatment and the sex factors. A 0 on the line means that no significant effect was observed, even if there was a tendency for a positive effect of the 10 min pretreatment in the 21/25 flies (see text). For the lifespan results, the results of the mutant and of its control are reported only when there is a significant interaction between the pretreatment and the genotype factors, and a 0 on the line means that no significant effect of the pretreatment was observed.

Genotype	Effect of heat pretreatment on resistance to lethal heat	Effect of heat pretreatment on lifespan
<i>w¹¹¹⁸</i>	0	Males, 5 min: -9.7%, 10 min: -7.2% Females, 5 min: -1.7%, 10 min: -3.5%
Meyzieu	0	0
Castanet	Males, 5 min: 0, 10 min: -5.8% Females, 5 min: +9.8%, 10 min: +12.9%	0
<i>dFOXO^{Δ94}</i>	5 min: +7.6%, 10 min: +24.8%	0
<i>dFOXO^{Δ94/25}</i>	Males, 5 min: 0, 10 min: +27.2% Females, 5 min: +9.3%, 10 min: +6.1%	5 min: +4.2%, 10 min: -12.6%
<i>dFOXO^{Δ94/+}</i> (control of <i>dFOXO^{Δ94/25}</i>)	Males, 5 min: 0, 10 min: +2.0% Females, 5 min: +6.2%, 10 min: +13.2%	5 min: -9.4%, 10 min: -2.2%
21/25	0	5 min: 0, 10 min: -4.5%
mix of 21/+ and 25/+	0	
<i>dFOXO^{Δ94/21}</i>	Males, 5 min: +10.9%, 10 min: +32.7% Females, 5 min: 0, 10 min: +5.1%	5 min: -1.1%, 10 min: -11.1%
<i>dFOXO^{Δ94/+}</i> (control of <i>dFOXO^{Δ94/21}</i>)	Males, 5 min: +4.9%, 10 min: +12.2% Females, 5 min: -8%, 10 min: +4.1%	

Figure 1. Mean survival time \pm SEM at 37 °C of 13-16 day-old wild-type flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age): each bar is the mean of 48 flies. A: Meyzieu flies. B: w^{1118} flies. C: Castanet flies.

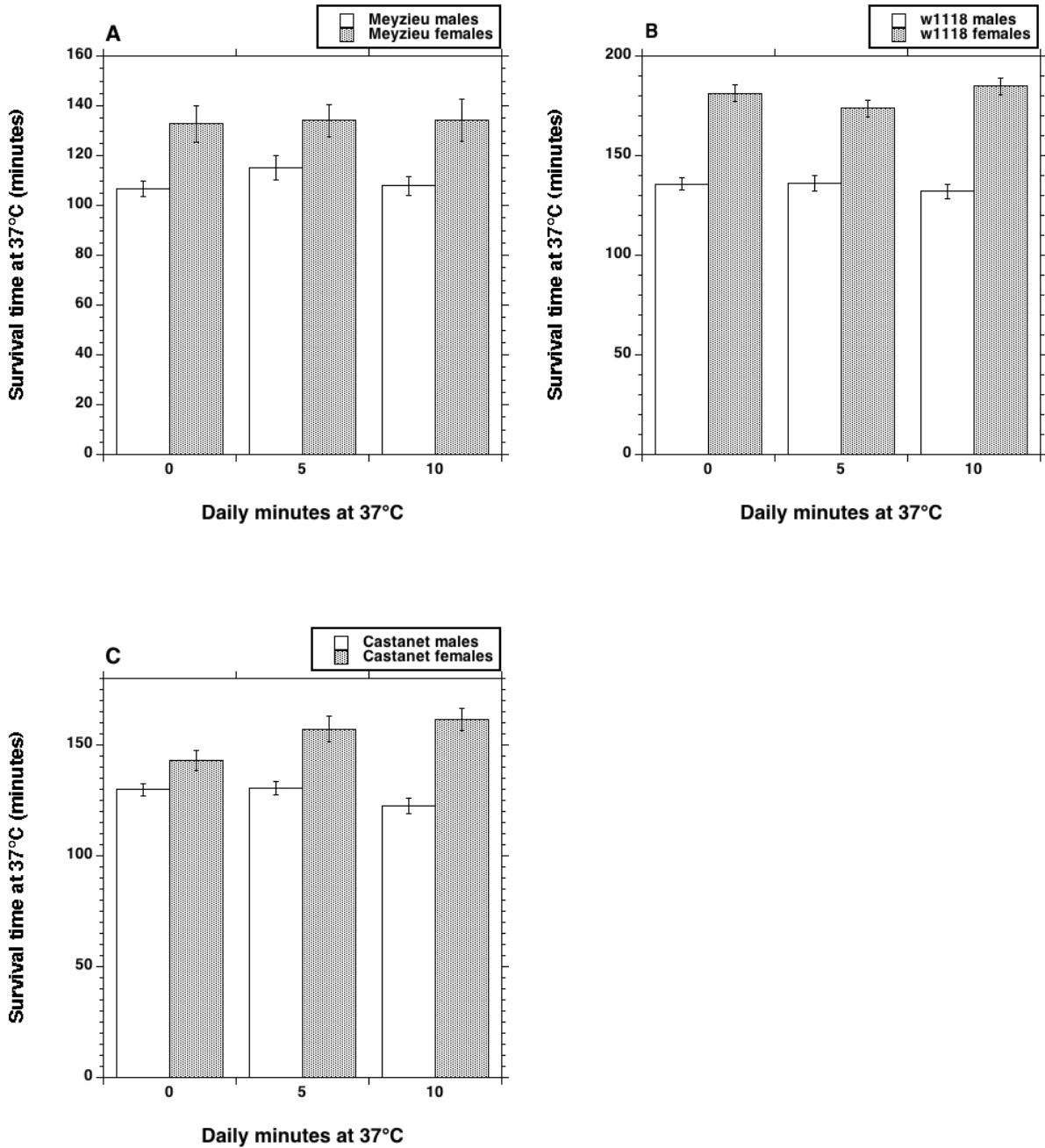
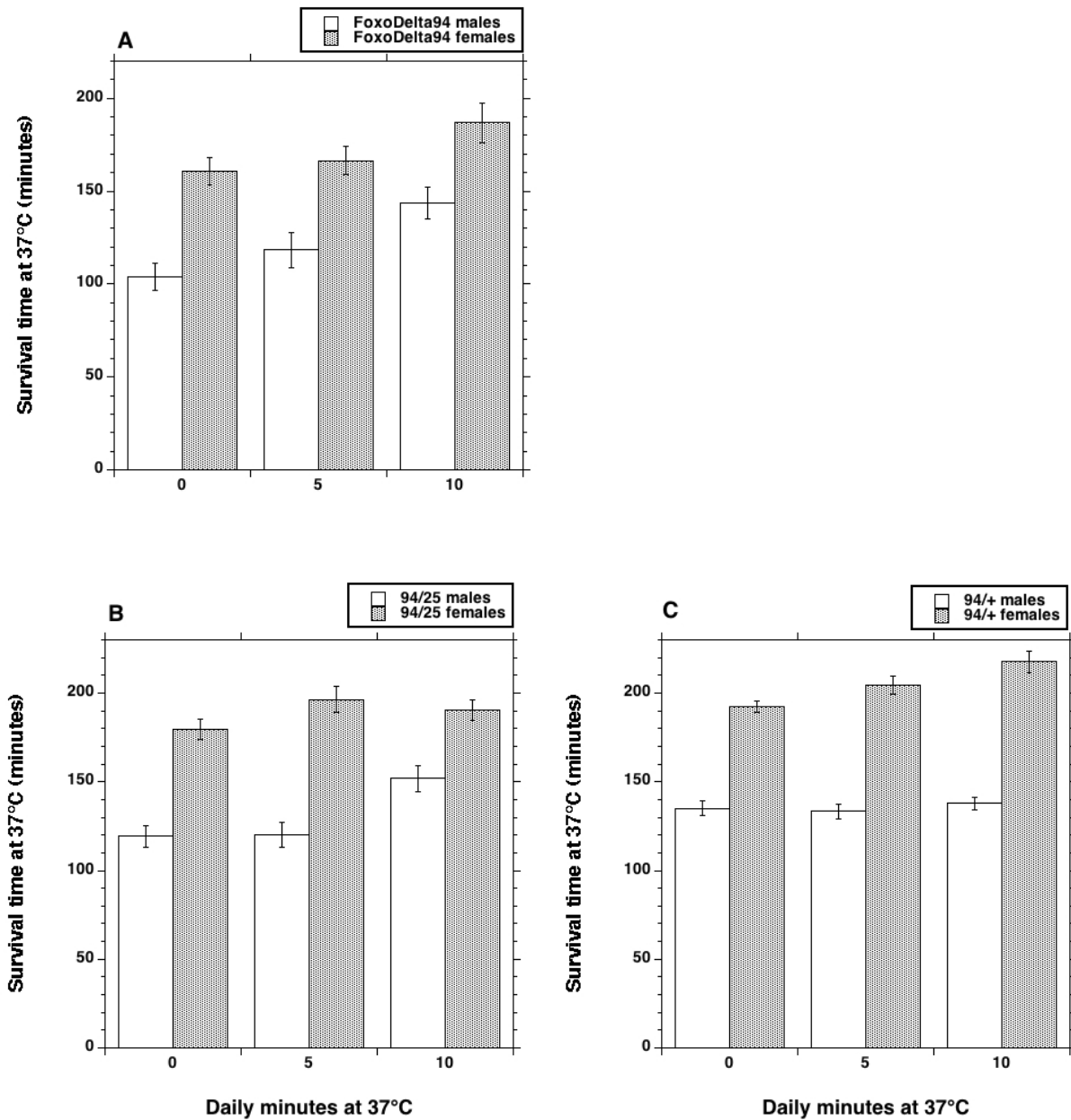


Figure 2. Mean survival time \pm SEM at 37 °C of 13-16 day-old *dFOXO* and control flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age): each bar is the mean of 48 flies, except for the D and E figures for which it is only 42 (see text for explanation). A: *dFOXO*^{*Δ94*} flies (FoxoDelta94 on the figure). B: *dFOXO*^{*Δ94/25*} flies (94/25 on the figure). C: *dFOXO*^{*Δ94/+*} (94/+ on the figure). D: 21/25 flies. E: mix of 21/+ and 25/+ (+ on the figure) flies. F: *dFOXO*^{*Δ94/21*} flies (94/21 on the figure). G: *dFOXO*^{*Δ94/+*} (94/+ on the figure, control of 94/21).



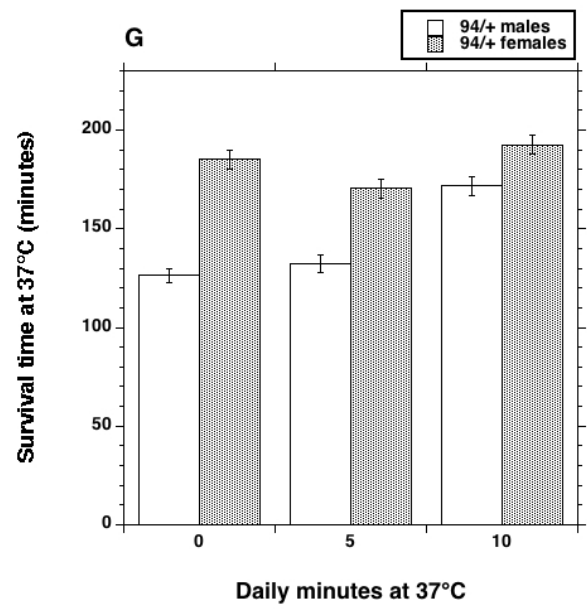
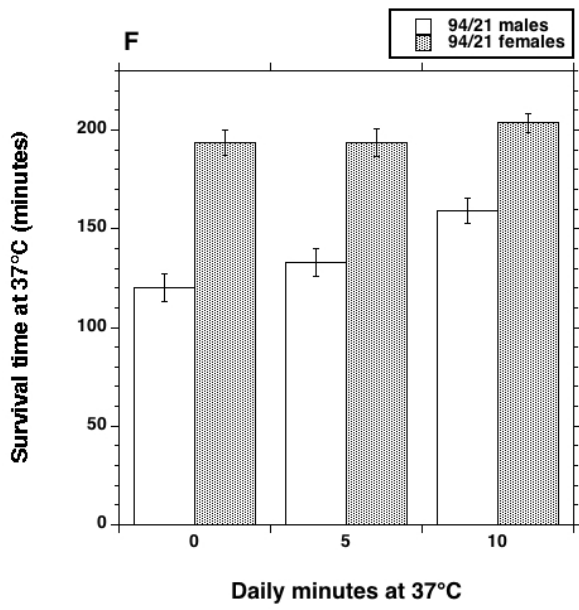
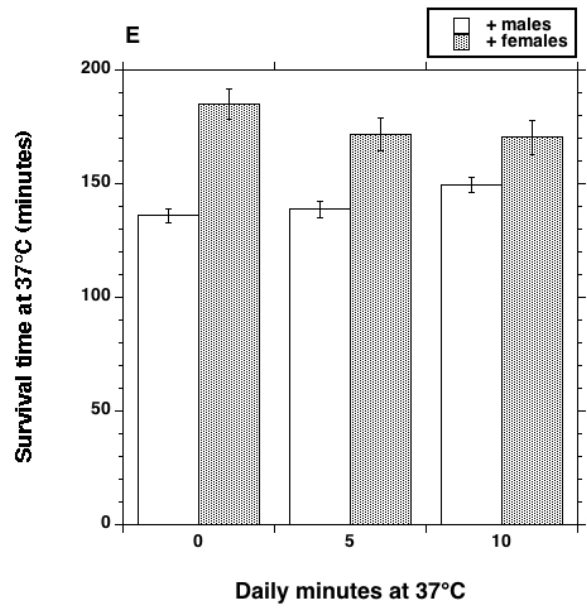
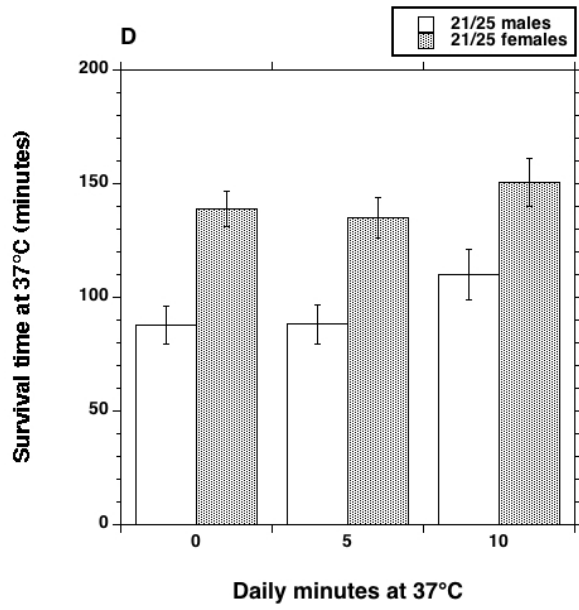


Figure 3. Mean survival time \pm SEM at 37 °C of 13-16 day-old flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age). The dFOXO group (Foxo on the figure) pools the four mutant genotypes (*dFOXO^{Δ94}*flies, 21/25, *dFOXO^{Δ94}*flies/25, *dFOXO^{Δ94}*flies/21 flies): each bar is the mean of 186 flies. The wild-type group (Wild on the figure) pools the six wild-type genotypes (*w¹¹¹⁸*, Meyzieu, Castanet, *dFOXO^{Δ94}*flies/+ (controls of *dFOXO^{Δ94}*flies/25 and *dFOXO^{Δ94}*flies/21), mix of 21/+ and 25/+ flies): each bar is the mean of 282 flies.

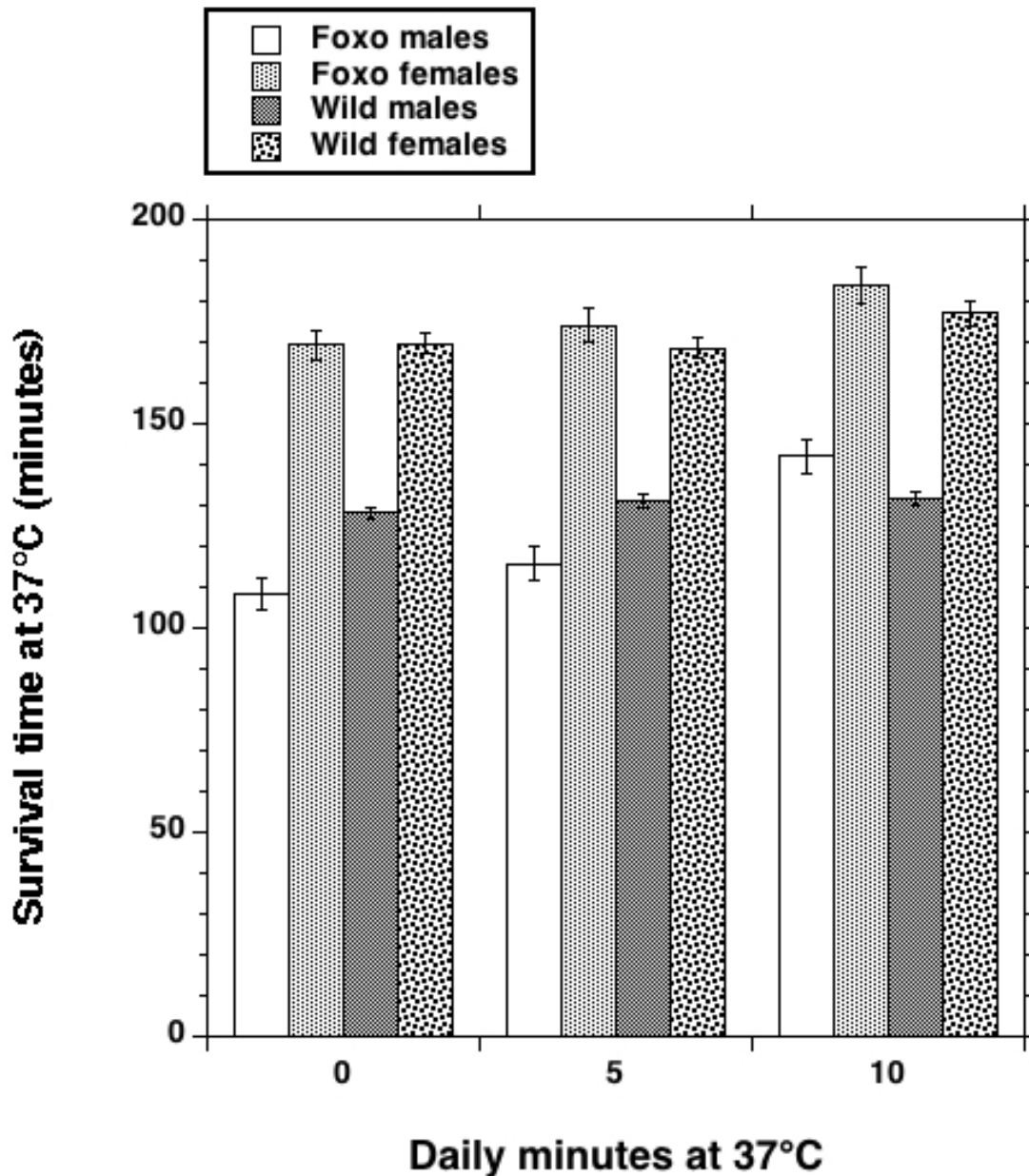
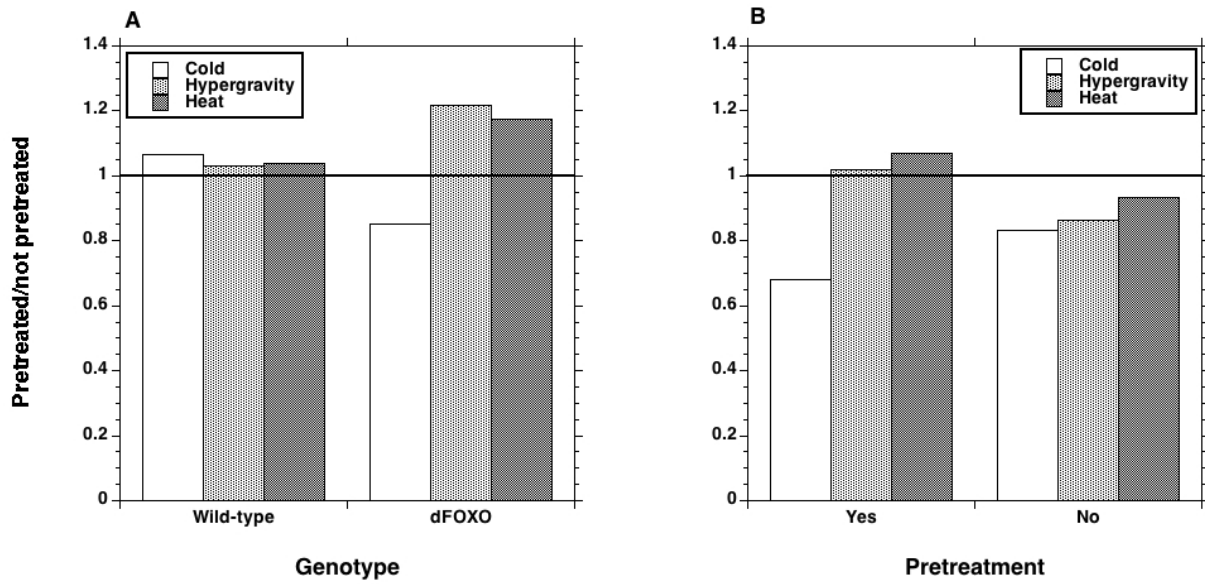


Figure 4. Comparison of the effects of the pretreatment and of the *dFOXO* mutation on survival time at 37 °C in flies subjected or not to cold, hypergravity or heat pretreatment at young age. For the hypergravity and heat pretreatments, the best result is taken into account (hypergravity: mean of 5 g groups, heat: mean of 10 minutes groups). A: ratio of the mean of each pretreated group on the not-pretreated one in the pool of all wild-type control or *dFOXO* flies. B: ratio of the mean of all *dFOXO* flies on wild-type groups ones in pretreated and not-pretreated groups.



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Supplementary material of

A mild heat stress increases resistance to heat of *dFOXO Drosophila melanogaster* mutants but less in wild-type flies

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Viability and sex-ratio data

Table S1. Viability and sex-ratio results of Meyzieu, *dFOXO⁴⁹⁴*, *w¹¹¹⁸* and Castanet flies used in the experiments \pm confidence interval (C.I.) at the $p = 0.05$ significance level. Viability is the percentage of imagoes divided by the number of harvested eggs and sex-ratio is the percentage of female imagoes. The date of emergence is indicated by the number of the week in the calendar year (e.g. the first week of 2019 is 1/2019).

Group (number of eggs)	Viability \pm C.I. (100 % expected)	Sex-ratio \pm C.I. (50 % expected)
	<i>Meyzieu</i>	
34/2019 (800)	71.13 \pm 3.14	50.97 \pm 4.11
35/2019 (350)	86.29 \pm 3.60	50.99 \pm 5.64
36/2019 (500)	86.00 \pm 3.04	53.26 \pm 4.72
	<i>dFOXO⁴⁹⁴</i>	
39/2019 (875)	25.83 \pm 2.90	52.21 \pm 6.51
40/2019 (700)	33.86 \pm 3.51	42.62 \pm 6.30
41/2019 (875)	27.43 \pm 2.96	54.58 \pm 6.30
42/2019 (875)	29.37 \pm 3.02	50.97 \pm 6.11
43/2019 (1050)	22.86 \pm 2.54	55.00 \pm 6.29
	<i>w¹¹¹⁸</i>	
01/2020 (1050)	75.62 \pm 2.60	51.51 \pm 3.48
02/2020 (525)	83.62 \pm 3.17	50.80 \pm 4.68
	<i>Castanet</i>	
22/2020 (350)	94.29 \pm 2.43	51.52 \pm 5.39
23/2020 (350)	92.57 \pm 2.75	50.00 \pm 5.44
24/2020 (350)	82.00 \pm 4.02	49.13 \pm 5.78
35/2020 (700)	84.86 \pm 2.66	50.00 \pm 4.02

Table S2. Viability and sex-ratio results of 21/25 and a mix of 21/+ and 25/+ flies used in the experiments \pm confidence interval (C.I.) at the $p = 0.05$ significance level. 25/+ females were crossed with 21/+ males. The maximal expected viability is 75%, one quarter of crosses being lethal because of the balancer chromosome.

Group (number of eggs)	Viability \pm C.I. (75 % expected)	Sex-ratio \pm C.I. (50 % expected)	Percentage of flies \pm C.I.		Sex-ratio \pm C.I.	
			21/25 (33 % expected)	21/+, 25/+ (67 % expected)	21/25 (50 % expected)	21/+, 25/+ (50 % expected)
04/2020 (1050)	53.52 \pm 3.02	54.27 \pm 4.12	29.36 \pm 3.77	70.64 \pm 3.77	59.39 \pm 7.49	52.14 \pm 4.91
06/2020 (1400)	50.79 \pm 2.62	48.52 \pm 3.67	33.33 \pm 3.47	66.67 \pm 3.47	53.59 \pm 6.35	45.99 \pm 4.49
07/2020 (1750)	52.91 \pm 2.34	50.97 \pm 3.22	31.43 \pm 2.99	68.57 \pm 2.99	51.55 \pm 5.74	50.71 \pm 3.89
09/2020 (1750)	52.46 \pm 2.34	51.31 \pm 3.23	28.00 \pm 2.90	72.00 \pm 2.90	49.42 \pm 6.11	52.04 \pm 3.81

Table S3. Viability and sex-ratio results of the offspring of the cross between *dFOXO^{Δ94}* males and 25/+ females \pm confidence interval (C.I.) at the $p = 0.05$ significance level. The offspring are the *dFOXO^{Δ94}/25* (94/25 in the table) and *dFOXO^{Δ94}/+* (94/+) flies used in experiments.

Group (number of eggs)	Viability \pm C.I. (100 % expected)	Sex-ratio \pm C.I. (50 % expected)	Percentage of flies \pm C.I. (50 % expected)		Sex-ratio \pm C.I. (50 % expected)	
			94/25	94/+	94/25	94/+
44/2019 (875)	70.63 \pm 3.02	48.54 \pm 3.94	46.60 \pm 3.93	53.40 \pm 3.93	47.92 \pm 5.77	49.09 \pm 5.93
45/2019 (700)	67.14 \pm 3.48	49.79 \pm 4.50	46.38 \pm 4.51	53.62 \pm 4.51	50.46 \pm 6.64	49.21 \pm 6.17
46/2019 (700)	64.43 \pm 3.55	57.21 \pm 4.57	44.57 \pm 4.59	55.43 \pm 4.59	57.71 \pm 6.83	56.80 \pm 6.14
47/2019 (350)	70.00 \pm 4.80	48.16 \pm 6.26	51.84 \pm 6.26	48.16 \pm 6.26	49.61 \pm 8.70	51.69 \pm 9.02
48/2019 (525)	68.76 \pm 3.96	45.15 \pm 5.13	52.91 \pm 5.15	47.09 \pm 5.15	44.50 \pm 7.05	45.88 \pm 7.49

Table S4. Viability and sex-ratio results of the offspring of the cross between *dFOXO⁴⁹⁴* males and 21/+ females \pm confidence interval (C.I.) at the $p = 0.05$ significance level. The offspring are the *dFOXO⁴⁹⁴/21* (94/21 in the table) and *dFOXO⁴⁹⁴/+* (94/+) flies used in experiments.

Group (number of eggs)	Viability \pm C.I. (100 % expected)	Sex-ratio \pm C.I. (50 % expected)	Percentage of flies \pm C.I. (50 % expected)		Sex-ratio \pm C.I. (50 % expected)	
			94/21	94/+	94/21	94/+
37/2020 (700)	68.14 \pm 3.45	50.94 \pm 4.49	50.31 \pm 4.49	49.69 \pm 4.49	48.75 \pm 6.32	53.16 \pm 6.35
45/2020 (525)	77.71 \pm 3.56	55.15 \pm 4.83	45.59 \pm 4.83	54.41 \pm 4.83	50.54 \pm 7.19	59.01 \pm 6.47
46/2020 (525)	75.43 \pm 3.68	48.74 \pm 4.92	51.26 \pm 4.92	48.74 \pm 4.92	46.31 \pm 6.85	51.30 \pm 7.05
47/2020 (525)	77.33 \pm 3.58	52.95 \pm 4.86	50.49 \pm 4.86	49.51 \pm 4.86	50.73 \pm 6.84	55.22 \pm 6.87
48/2020 (525)	62.67 \pm 4.14	52.89 \pm 5.39	44.38 \pm 5.37	55.62 \pm 5.37	47.94 \pm 8.10	56.83 \pm 7.18

Table S5: ANOVA of the effects of sex, pretreatment, genotype and of the FOXO genotype on survival time to heat. The genotype factor (*dFOXO⁴⁹⁴*, *dFOXO⁴⁹⁴/25*, *dFOXO⁴⁹⁴/21*, Meyzieu, *w¹¹¹⁸*, Castanet, *dFOXO⁴⁹⁴/+* (controls of *dFOXO⁴⁹⁴/25* and *dFOXO⁴⁹⁴/21*), mix of 21/+ and 25/+) is considered as random and nested in the FOXO genotype factor.

Source	Degrees of freedom	Sum of squares	Mean square	F-ratio	p-value
Sex (1)	1	1507280.00	1507280.00	106.76	≤ 0.0001
Pretreatment (2)	2	105618.00	52809.10	31.28	≤ 0.0001
(1) by (2)	2	6640.56	3320.28	1.38	0.2793
Genotype (3)	8	785836.00	98229.50	56.19	≤ 0.0001
(1) by (3)	8	112943.00	14117.90	8.08	≤ 0.0001
(2) by (3)	16	27013.10	1688.32	0.97	0.4921
(1) by (2) by (3)	16	38421.10	2401.32	1.37	0.1449
FOXO genotype (4)	1	6748.05	6748.05	0.07	0.7999
(1) by (4)	1	24198.70	24198.70	1.71	0.2268
(2) by (4)	2	41357.50	20678.70	12.25	0.0006
(1) by (2) by (4)	2	19466.90	9733.45	4.05	0.0377
Error	2748	4803984	1748.17		
Total	2807	7456537			

Figure S1. Survival curves at 37 °C of 13-16 day-old wild-type flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age): n = 48 for each curve. A: Meyzieu flies. B: *w¹¹¹⁸* flies. C: Castanet flies.

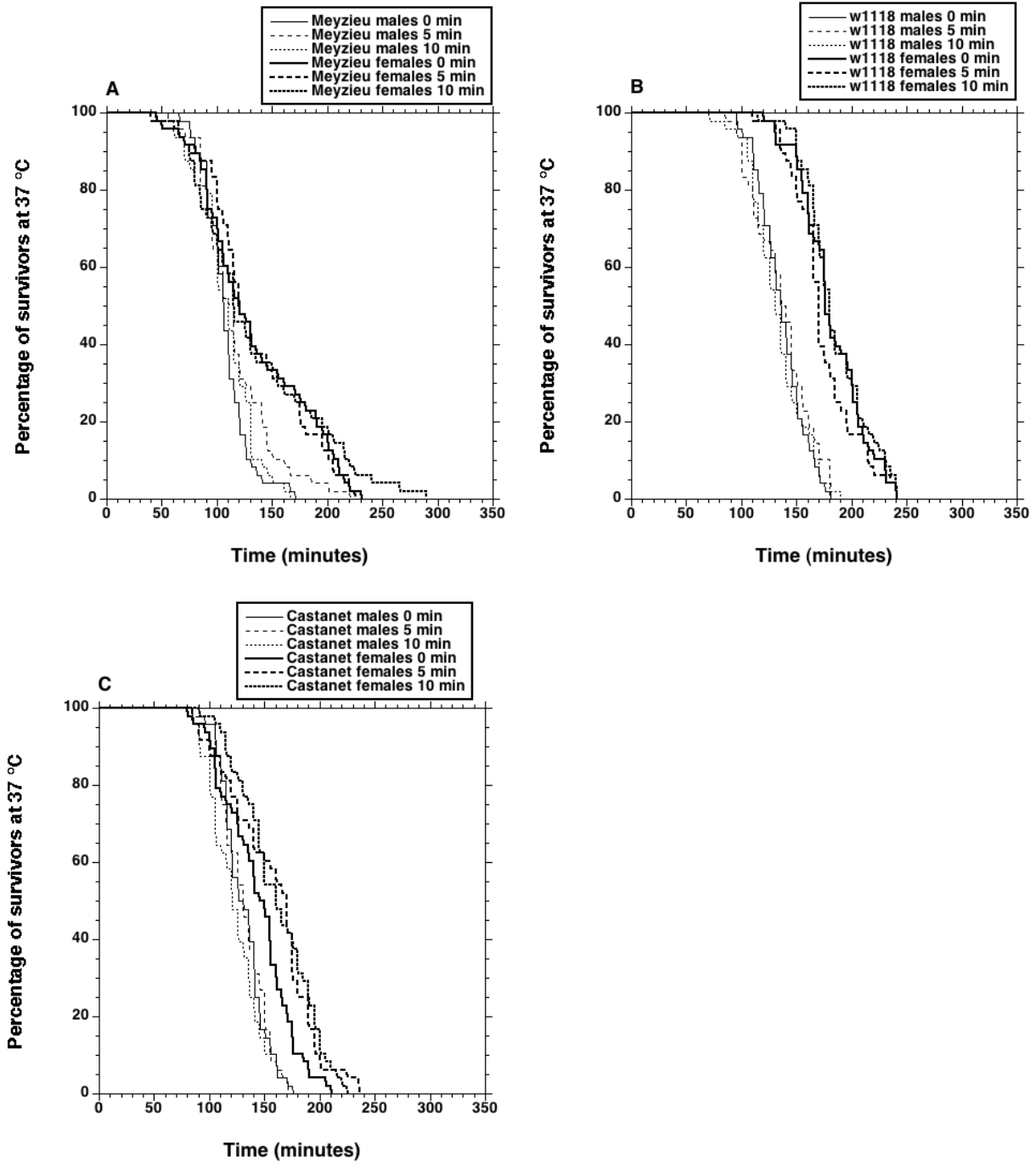
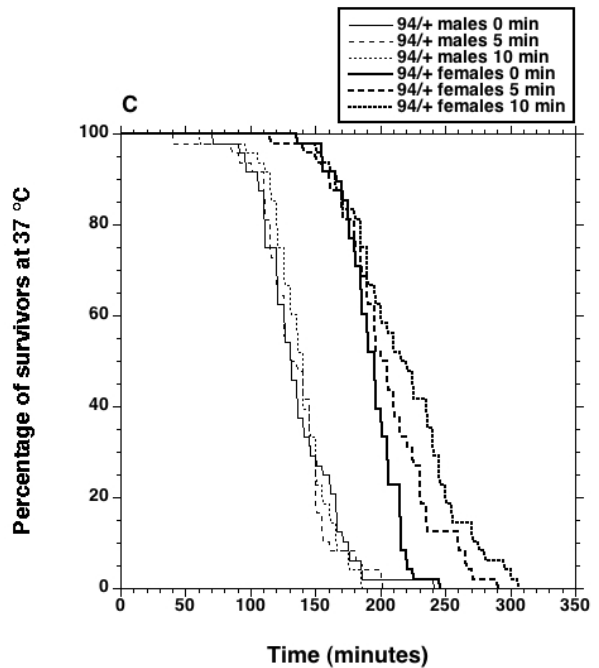
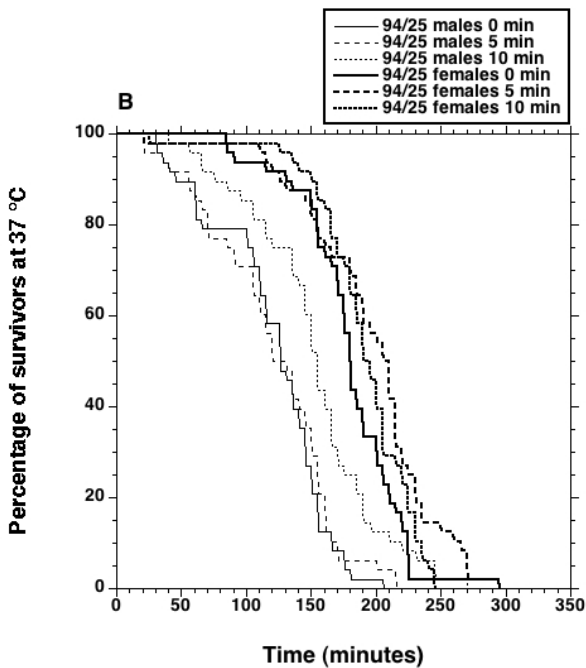
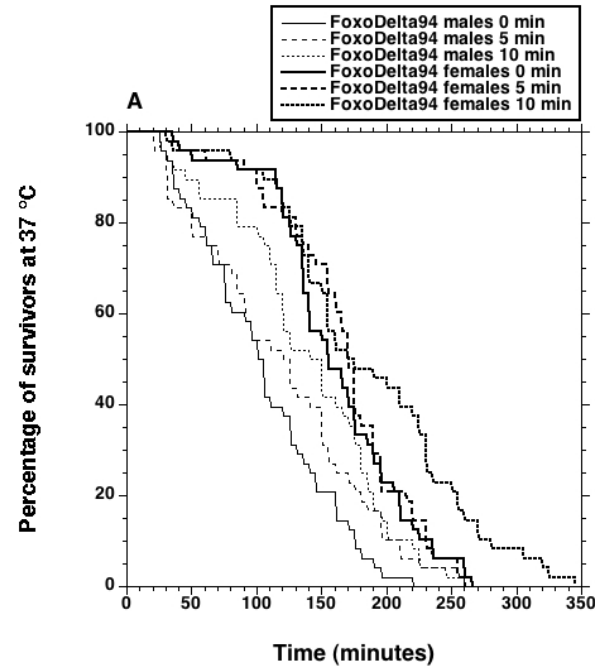


Figure S2. Survival curves at 37 °C of 13-16 day-old *dFOXO* and control flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age): n = 48 for each curve, except for the D and E figures for which it is only 42 (see text for explanation). A: *dFOXO*^{A94} flies (FoxoDelta94 on the figure). B: *dFOXO*^{A94/25} flies (94/25 on the figure). C: *dFOXO*^{A94/+} (94/+ on the figure). D: 21/25 flies. E: mix of 21/+ and 25/+ (+ on the figure) flies. F: *dFOXO*^{A94/21} flies (94/21 on the figure). G: *dFOXO*^{A94/+} (94/+ on the figure, control of 94/21).



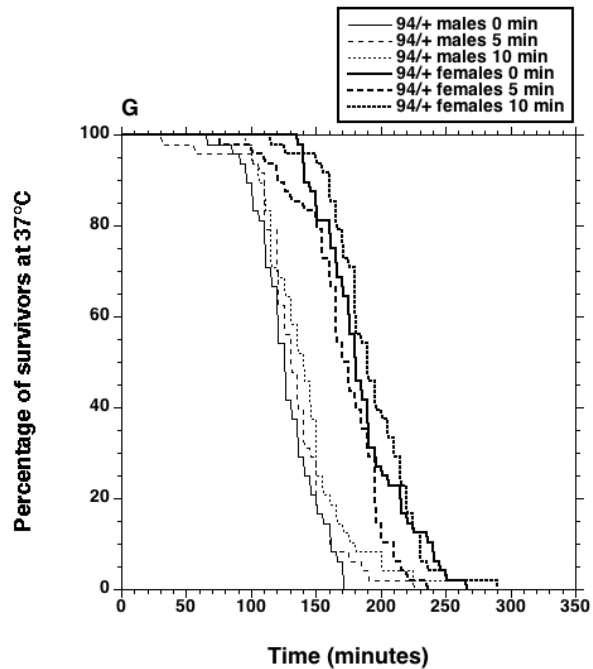
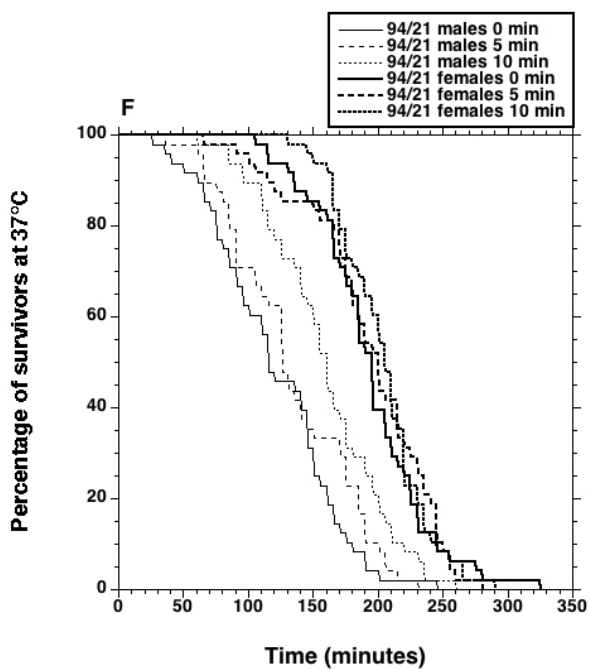
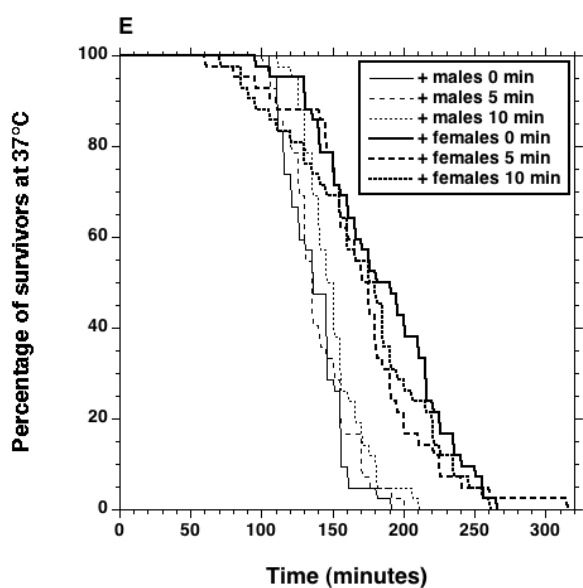
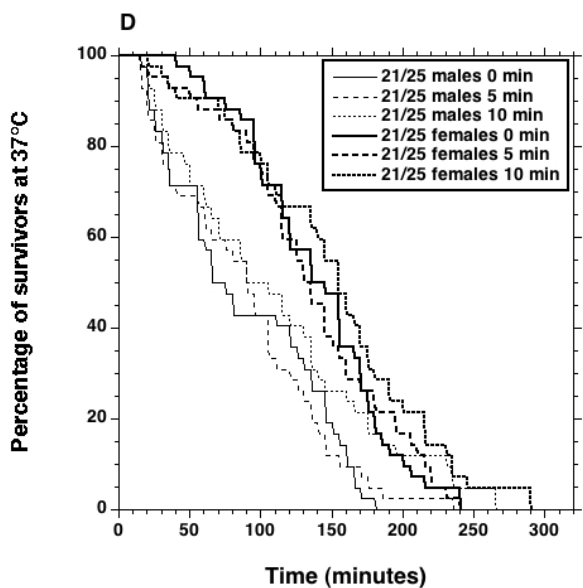
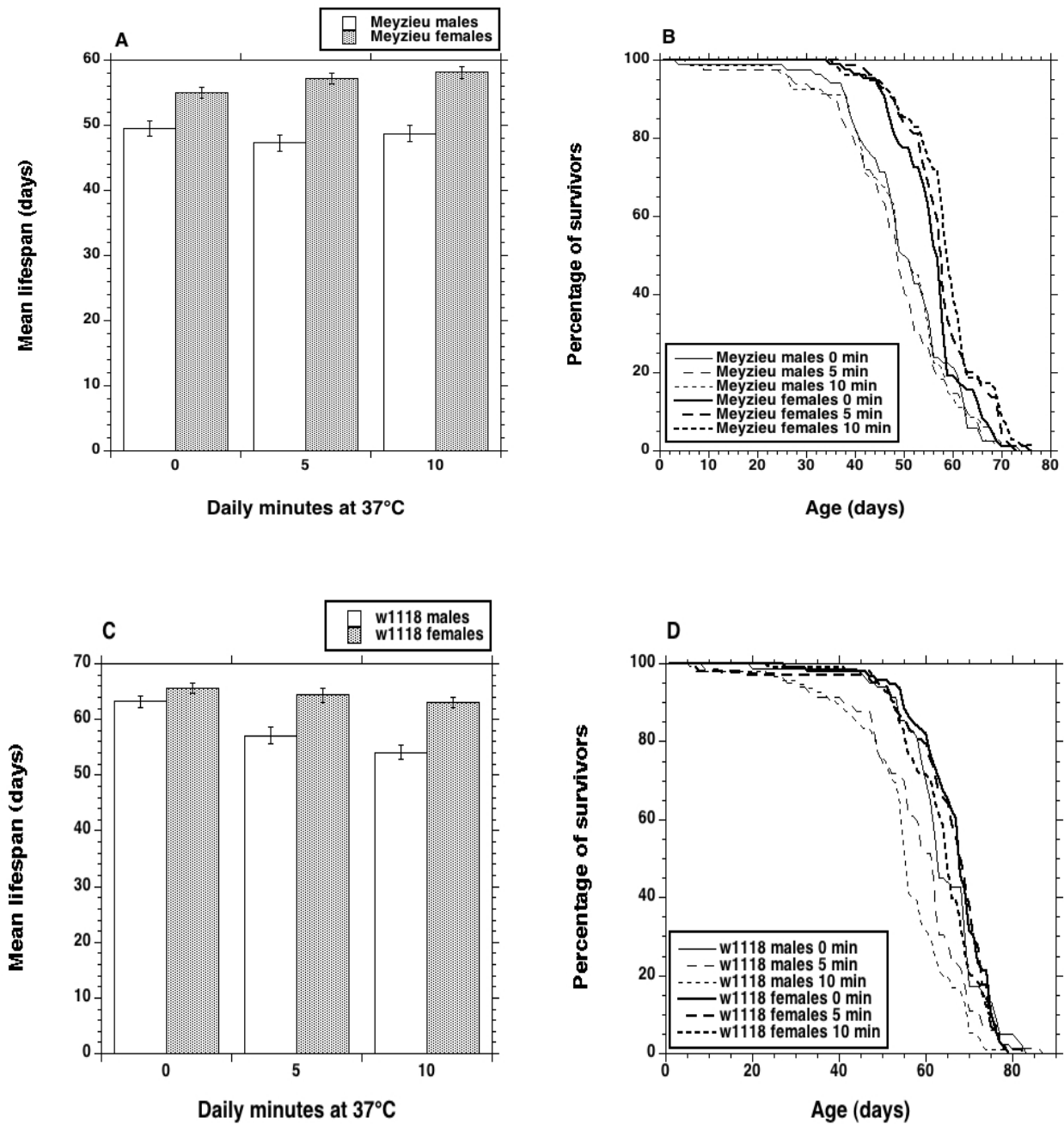


Figure S3. Lifespan of wild-type flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age). Meyzieu flies: A: mean lifespan \pm SEM, each bar is the mean of 75-84 flies; B: longevity curves. *w¹¹¹⁸* flies: C: mean lifespan \pm SEM, each bar is the mean of 82-102 flies; D: longevity curves. Castanet flies: E: mean lifespan \pm SEM, each bar is the mean of 76-84 flies; F: longevity curves.



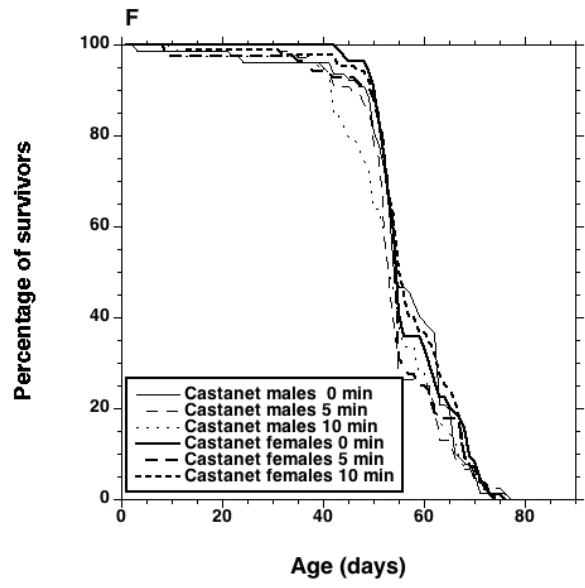
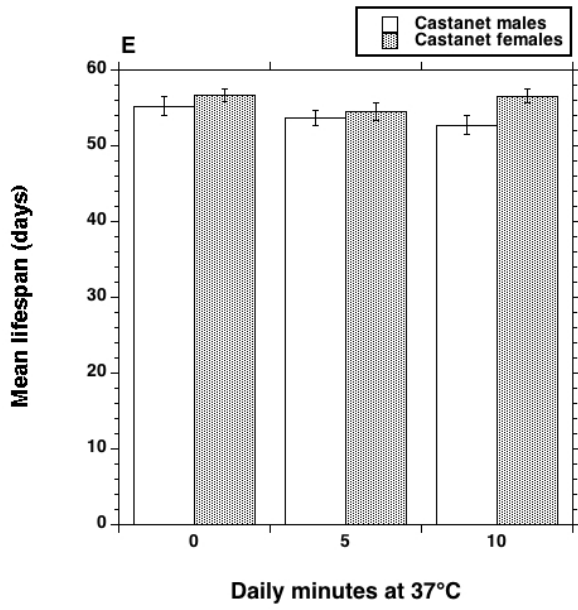


Figure S4. Lifespan of *dFOXO* and control flies subjected or not to a heat pretreatment at young age (0, 5, or 10 min at 37 °C daily from 5 to 9 days of age). *dFOXO^{Δ94}* flies (FoxoDelta94 on the figure): A: mean lifespan ± SEM, each bar is the mean of 65-90 flies; B: longevity curves. *dFOXO^{Δ94}/25* flies (94/25 on the figure): C: mean lifespan ± SEM, each bar is the mean of 24-37 flies; D: longevity curves. *dFOXO^{Δ94}/+* flies (94/+ on the figure): E: mean lifespan ± SEM, each bar is the mean of 24-42 flies; F: longevity curves. 21/25 flies: G: mean lifespan ± SEM, each bar is the mean of 30-56 flies; H: longevity curves. Mix of 21/+ and 25/+ flies (+ on the figure): I: mean lifespan ± SEM, each bar is the mean of 110-146 flies; J: longevity curves. *dFOXO^{Δ94}/21* flies (94/21 on the figure): K: mean lifespan ± SEM, each bar is the mean of 23-44 flies; L: longevity curves. *dFOXO^{Δ94}/+* flies (94/+ on the figure, control of 94/21): M: mean lifespan ± SEM, each bar is the mean of 23-42 flies; N: longevity curves.

