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RESEARCH ARTICLE

Hypergravity increases resistance to heat in *dFOXO* *Drosophila melanogaster* mutants and can lower FOXO translocation in wild-type males

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Abstract Severe stresses have deleterious effects, but mild stresses can have beneficial effects called hormetic effects. This study observed survival time of wild-type *Drosophila melanogaster* flies and *dFOXO* mutants exposed to 37 °C, a severe stress for flies, after they lived or not for 2 weeks in hypergravity (3 or 5 g), a mild stress with hormetic effects in flies. Hypergravity increased survival time of the mutants, this effect being less observed in wild-type flies. 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 the gravity level being observed in females. Because hypergravity increases resistance to heat in *dFOXO* mutants and the translocation is not tightly dependent on the gravity level, one can conclude that dFOXO does not mediate the effect of hypergravity on resistance to heat. A previous study showed that another mild stress, the cold, can increase survival time at 37 °C of wild-type *D. melanogaster* flies, but this was not observed in *dFOXO* mutants. 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.

Keywords Mild stress · Hypergravity · Heat stress · dFOXO mutants · *Drosophila melanogaster*

Introduction

Although any severe stress can have deleterious effects, it is now well established that mild stresses can have beneficial effects: these beneficial effects are called hormetic effects (Mattson and Calabrese 2010). A mild stress, i.e. a low intensity stimulus disturbing the homeostasis of the organism without inducing severe damages, can provoke adaptive responses enhancing the ability to resist severe stresses at young or old age and increase lifespan. These results can be observed in flies, nematodes, mammals, and

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particularly in human beings (reviews in e.g. Le Bourg 2009; Rattan and Le Bourg 2014; Rattan and Kyriazis 2019).

Having established that mild stresses can have beneficial effects, the next step on the research agenda is to know the mechanisms explaining these effects. Studies in *Drosophila melanogaster* have shown that the antioxidant enzymes superoxide dismutase and catalase are probably not involved (Le Bourg and Fournier 2004), that 70 kDa heat-shock proteins (HSP70) could explain, among other mechanisms, the better resistance to heat after a mild stress (Kristensen et al. 2003; Le Bourg et al. 2002; Sørensen et al. 2007), and that the NF-κB-like transcription factor DIF (dorsal-related immunity factor) could explain the better resistance to fungi and heat after a mild cold stress, but not that to a severe cold stress (Le Bourg et al. 2012).

The Forkhead box class O (FOXO) transcription factor, a downstream effector of the insulin/insulin-like growth factor-1 pathway, is involved in resistance to stress in *D. melanogaster* (review in Puig and Mattila 2011) and *FOXO* mutant flies (*dFOXO*) could help to know whether FOXO explains the positive effects of mild stress on resistance to severe stress. Recently, we showed that a mild cold stress did not increase survival time at 37 °C of two *dFOXO* mutants, contrarily to what was observed in wild-type flies (Polesello and Le Bourg 2017). This heat stress strongly increased dFOXO nuclear translocation, but this effect was lowered 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 cold-pretreated flies, it seems that, although dFOXO is required to resist heat, other mechanisms partly substitute to dFOXO translocation in cold-pretreated flies.

However, these effects of dFOXO were observed in flies subjected to a mild cold stress and it would be of interest to know whether similar effects are observed with another mild stress. This is of importance because fasting, a mild stress increasing resistance of wild-type flies to a severe cold stress (Le Bourg 2013), can also—slightly—increase that of *dFOXO* mutants (Le Bourg and Massou 2015), but a mild irradiation does not increase tolerance of the same mutants to a strong irradiation, contrarily to what is observed in wild-type flies (Moskalev et al. 2011). Therefore, knowing whether dFOXO has similar effects when different mild stresses are used could allow to conclude whether dFOXO has a general relevance as a mechanism explaining hormetic effects.

Hypergravity (HG), i.e. gravity levels higher than 1 g, the Earth gravity level, can be considered as a stress because being subjected to 3 g means that the weight is magnified thrice. HG is not a natural stress, even if humans can experience short HG episodes in cars or elevators during strong braking. HG is used in mammals and other species since the 1960s, particularly in space research (review in Le Bourg 1999), and is also used in *D. melanogaster* to study the effects of mild stress on ageing and longevity (review in Le Bourg 2009).

Because HG is a mild stress known to increase resistance to heat (e.g. Le Bourg and Minois 1997), we decided to test whether HG (3 or 5 g) increases resistance to this severe stress in *dFOXO* mutants. Meyzieu wild-type flies were used to test whether HG and heat modulate nuclear translocation of the dFOXO protein.

Materials 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 were transferred under ether anaesthesia in groups of 15 flies of the same sex to 20 ml polystyrene vials. Flies were transferred to new vials twice a week. Except for the first 2 weeks of adult life, during which flies were kept in the room of the centrifuge (see below), flies spent their life in an incubator. In both the room of the centrifuge and the incubator the rearing temperature was 25 ± 0.5 °C and light was on from 07.00 to 19.00 h.

Meyzieu, w^{1118}, and $dFOXO^{\Delta 94}$ flies

The wild-type strain Meyzieu, caught at the end of the 1970s near the city of Lyon (France), the white-eye inbred wild-type strain w^{1118} (Hazelrigg et al. 1984), and the white-eye null mutant $dFOXO^{\Delta 94}$ (Slack et al. 2011) were used in this study. In order to obtain the

parents of the experimental flies, flies of the different strains were allowed to lay eggs for one night in a bottle. Flies emerging 9–10 days after egg-laying were transferred to fresh bottles: they 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 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} (25/+) and *dFOXO*^{21} (21/+) heterozygotes 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$^{\Delta 94}$ flies

25/+ flies were crossed with *dFOXO*$^{\Delta 94}$ ones to complete the results obtained with the 21/25 and *dFOXO*$^{\Delta 94}$ flies. Thus, *dFOXO* flies were *dFOXO*$^{\Delta 94}$/25 and heterozygotes were *dFOXO*$^{\Delta 94}$/+ . Offspring of the cross between *dFOXO*$^{\Delta 94}$ dams and 25/+ sires had a ca 30% viability, while offspring of the reciprocal cross had a ca 65% viability (viability and sex-ratio data are reported in the Supplemental Material section, Table S3). In addition, male heterozygotes of the reciprocal cross had a yellow body (*y*), while all the other flies of the two crosses were of a wild-type phenotype. Offspring of the two reciprocal crosses were used in the reported experiments.

Hypergravity pretreatment

HG is achieved by putting flies in a continuously rotating centrifuge (102 rpm, see Picture S1 in the Supplemental Material section). Flies were subjected to 3 or 5 g for 15 days from the second day of adult life. Flies not subjected to HG were placed near the rotating centrifuge during this time and thus subjected to the same environment, except the increased gravity level. After the period of centrifugation, all flies were transferred into the incubator described above.

Resistance to heat

The survival time in a water-bath set at 37 °C was observed at 20–23 days of age, thus ca 1 week after flies were transferred to the incubator. Flies were transferred in early morning, just before the heat shock, in groups of three 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 °C, the plug containing absorbent cotton with 65 ml of distilled water to prevent desiccation (12 vials in the water-bath). Vials were observed with a headset magnifier every 5 min: flies totally immobile during six successive records were considered to be dead. The experimenter (first author) could identify the gender and genotype when observing flies but was blind to the gravity group.

For the 21/25 and the mix of 21/+ and 25/+ flies, flies of the 38/2017 (i.e. week 38 in 2017), 40/2017, 47/2017, and 48/2017 groups were observed, for a total of 18 flies in each sex, genotype and gravity group (total n = 216). For the *dFOXO*$^{\Delta 94}$ strain, flies of the 18/2017, 19/2017, and 21/2017 groups were observed, for a total of 31 flies in each sex and gravity group (n = 186). For the *w*^{1118} strain, flies of the

51/2016, 52/2016, 02/2017, and 06/2017 groups were observed, for a total of 33 flies in each sex and gravity group (n = 198). For the reciprocal crosses between $dFOXO^{\Delta 94}$ and 25/+ flies, flies of the 03/2018, 04/2018, 05,2018, 06/2018, and 07/2018 were observed, for a total of 18 flies in each sex, gravity, genotype and reciprocal cross group (n = 432). Finally, for the Meyzieu strain, flies of the 01/2018, 02/2018, 03/2018, 10/2018, and 11/2018 groups were observed for a total of 84 flies in each sex and gravity group (n = 504).

For each experiment, survival times were analysed with an analysis of variance (ANOVA) testing the effect of sex, gravity level, and their interaction. When there was two genotypes, the genotype factor and all interactions were included in the design. Preliminary analyses showed that the way of the reciprocal cross between $dFOXO^{\Delta 94}$ and 25/+ flies had no real effect on survival time and this factor was thus not included in the reported results. In a second step of the analysis (see below), the effect of gravity, genotype, sex, and all their interactions was analysed separately in wild-type flies and mutants.

Nuclear translocation of dFOXO in Meyzieu flies

Flies of the Meyzieu strain (01/2019 and 18/2019 groups) were subjected or not to a 37 °C shock for 30 min at 21 days of age (5 days after the period of centrifugation) in a water-bath 30–60 min before being killed and dissected. Twelve flies in each sex, gravity level (1, 3 or 5 g), heat shock group (heat shock or not) were dissected in 1X phosphate buffered saline (PBS), fixed in 4% formaldehyde (Electron Microscopy Sciences) in PBS for 30 min at room temperature, and washed in PBS containing 0.1% Triton X-100 (PBT). Tissues were then blocked for 30 min in PBT containing 5% goat serum. The primary anti-Foxo antibody (a kind gift of Pierre Léopold, Slaidina et al. 2009) used in a previous study (Polesello and Le Bourg 2017) was incubated (1/500) overnight at 4 °C. AlexaFluor-555 secondary antibodies (Molecular Probes) were incubated for 2 h at room temperature at 1/500. TO-PRO-3 (Molecular Probes, 1/2000) was used to stain the nuclei for 30 min. After washes, tissues were mounted in Vectashield (Vector). Fluorescence images were acquired with a Leica SPE confocal laser scanning microscope (objective 40, zoom 1). The first author prepared blocks of images showing nuclei and nuclei with dFOXO and the second author, blind of the gravity and heat shock group of each block of images (the visceral body is different in males and females), counted nuclei in abdominal fat body (for each fly, left part of the picture on Fig. S1) and, among them, those containing dFOXO (right part of the picture on Fig. S1) with the software ImageJ (imagej.nih. gov/ij). The number of nuclei containing dFOXO or not was analysed with logistic models testing for the effect of gravity group, sex, heat shock, and all interactions.

Results

Resistance to heat

In a first step of the analysis, we tested whether the gravity level had any effect on survival to heat in the different genotypes. Table 1 summarises the results and Figure S1 (Supplementary material) shows the results of each sex, gravity and genotype group.

Females of the w^{1118} strain better resisted than males (Fig. S1a, $F(1, 192) = 146.36$, $p < 0.0001$; mean ± SEM: 187.27 ± 4.14 vs 131.31 ± 1.98 min). The gravity level and its interaction with sex had no significant effect (Fs < 1). Thus, HG did not help w^{1118} flies to survive a severe heat stress.

Table 1 Summary of the effects of HG on survival time at 37 °C observed in each mutant or wild-type genotype

Genotype	Effect of HG on resistance to heat
w^{1118}	0
Meyzieu	3 g: +4.7%, 5 g: +7.9%
$dFOXO^{\Delta 94}$	Males: 3 g: 0, 5 g: +63.9%
	Females: 3 g: +10.0%, 5 g: +6.1%
21/25	0
Mix of 21/+ and 25/+	0
dFOXOΔ94/25	3 g: 0, 5 g: +16.4%
dFOXOΔ94/+	0

When a significant effect of HG is observed, the percentage of increase for 3 g and 5 g flies compared to 1 g ones is indicated, a 0 meaning less than a 1% variation. A 0 on the line means that no significant effect was observed, even if there was a tendency for a positive effect of HG in the 21/25 male flies (see text). The results of the two sexes are reported when there is a significant interaction between the gravity and the sex factors

Females of the Meyzieu wild-type strain better resisted than males (Fig. S1b, F(1, 498) = 195.57, $p < 0.0001$; 140.71 ± 2.20 vs. 105.66 ± 1.23 min). The survival time slightly increased with the gravity level (F(2, 498) = 4.69, p = 0.0096; 1, 3, and 5 g groups, respectively: 118.21 ± 2.47, 123.78 ± 2.65, 127.56 ± 2.56 min). The gravity by sex interaction had no significant effect (F close to 1). Thus, HG slightly increased survival to heat in both sexes of this wild-type strain.

Females of the *dFOXO*$^{\Delta 94}$ strain slightly better resisted than males (Fig. S1c, F(1, 180) = 3.97, p = 0.0479; 131.34 ± 5.74 vs. 113.44 ± 7.41 min). The gravity level, mainly at 5 g, increased survival time (F(2, 180) = 5.26, p = 0.0060; 1, 3, and 5 g groups, respectively: 109.03 ± 7.41, 115.48 ± 8.60, 142.66 ± 7.96 min) but the gravity by sex interaction showed that this effect was mainly due to 5 g males (F(2, 180) = 4.78, p = 0.0095; 1, 3, and 5 g females: 124.68 ± 9.00, 137.10 ± 10.34, 132.26 ± 10.60 min; males: 93.39 ± 11.23, 93.87 ± 12.75, 153.07 ± 11.75 min). Thus, 5 g *dFOXO*$^{\Delta 94}$ males better survived a severe heat stress and a lower positive effect of HG was observed in females.

Females of the *dFOXO*$^{\Delta 94}$/25 and *dFOXO*$^{\Delta 94}$/+ genotypes also better resisted than males (Fig. S1d, F(1, 420) = 366.53, $p < 0.0001$; 201.81 ± 2.61 vs. 123.68 ± 3.53 min). The *dFOXO*$^{\Delta 94}$/+ flies better resisted than the *dFOXO*$^{\Delta 94}$/25 ones (F(1, 420) = 38.87, $p < 0.0001$; 175.46 ± 3.02 vs. 150.02 ± 4.78 min) and the sex by genotype interaction showed that this effect was mainly due to males (F(1, 420) = 18.34, $p < 0.0001$; *dFOXO*$^{\Delta 94}$/25 and *dFOXO*$^{\Delta 94}$/+ females, respectively: 197.82 ± 3.90 vs. 205.79 ± 3.45 min; males: 102.22 ± 5.81 vs. 145.14 ± 2.77 min). Flies kept at 5 g better resisted than those at 3 g or that always lived at 1 g (F(2, 420) = 4.00, p = 0.0190; 1, 3, and 5 g groups, respectively: 158.82 ± 5.18, 158.51 ± 4.88, 170.90 ± 4.91 min) and the gravity by genotype interaction showed that this effect was only due to *dFOXO*$^{\Delta 94}$/25 flies (F(2, 420) = 3.16, p = 0.0436; 1, 3, and 5 g *dFOXO*$^{\Delta 94}$/25 flies: 142.08 ± 7.95, 142.57 ± 8.22, 165.42 ± 8.46 min; *dFOXO*$^{\Delta 94}$/+ flies: 175.56 ± 6.09, 174.44 ± 4.62, 176.39 ± 4.97 min). The other interactions were not significant. Separate analyses for each genotype confirmed that no gravity effect was observed in *dFOXO*$^{\Delta 94}$/+ flies, but was present in *dFOXO*$^{\Delta 94}$/25 ones (data not shown). Thus a positive effect of HG was observed in *dFOXO*$^{\Delta 94}$/25 flies but not in *dFOXO*$^{\Delta 94}$/+ ones.

For the 21/25 and the mix of 21/+ and 25/+ flies, females better resisted than males (Fig. S1e, F(1, 204) = 155.63, $p < 0.0001$; 182.59 ± 4.50 vs. 108.10 ± 5.08 min). The 21/25 flies survived for a lower time than heterozygotes (F(1, 204) = 49.89, $p < 0.0001$; 124.26 ± 6.87 vs. 166.44 ± 4.07 min) and the sex by genotype interaction showed that this effect was more important in males (F(1, 204) = 15.21, $p < 0.0001$; 21/25 and heterozygote females, respectively: 173.15 ± 7.14 vs. 192.04 ± 5.24 min; males: 75.37 ± 7.01 vs. 140.83 ± 3.84 min). The gravity effect and the other interactions were not significant (Fs close to or lower than 1), even if inspection of Fig. S1e shows a slight tendency for a better resistance in flies that have lived in HG (general means at 1, 3 and 5 g: 138.75 ± 7.99, 146.11 ± 6.64, 151.18 ± 7.33 min), this tendency being more striking in 21/25 males (means at 1, 3 and 5 g: 56.11 ± 10.55, 82.50 ± 12.44, 87.50 ± 12.66 min). Thus, a positive effect of HG is not

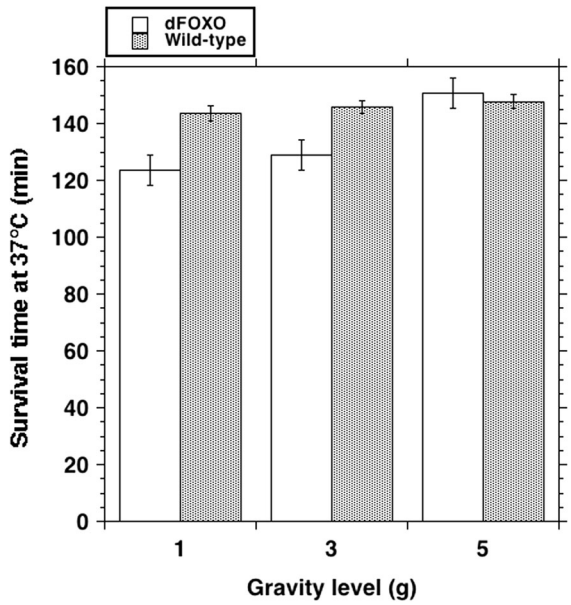

Fig. 1 Survival to heat. Mean survival time ± SEM at 37 °C of *dFOXO* and wild-type flies kept or not in HG for 2 weeks at young age. The *dFOXO* group pools the three mutant genotypes (*dFOXO*$^{\Delta 94}$, 21/25, *dFOXO*$^{\Delta 94}$/25 flies): each bar is the mean of 170 flies. The wild-type group pools the four wild-type genotypes (w^{1118}, Meyzieu, *dFOXO*$^{\Delta 94}$/+ , mix of 21/+ and 25/+ flies): each bar is the mean of 342 flies

clearly observed in this experiment in these *dFOXO* or control flies.

Table 1 summarises the results: it seems that *dFOXO* flies (21/25, $dFOXO^{\Delta 94}$, $dFOXO^{\Delta 94}$/25) from the HG groups survived longer than 1 g ones, this effect being not clearly observed in control sibling and wild-type flies (w^{1118}, $dFOXO^{\Delta 94}$/+ , mix of 21/+ and 25/+) except in Meyzieu flies.

In a second step of the analysis, *dFOXO* (21/25, $dFOXO^{\Delta 94}$, $dFOXO^{\Delta 94}$/25) and control flies (Meyzieu, w^{1118}, $dFOXO^{\Delta 94}$/+ , mix of 21/+ and 25/+) were analysed in separate ANOVAs (factors: sex, genotype, gravity and all interactions) to clarify the effect of the *dFOXO* loss of function mutation. Figure 1 shows the results of each gravity group for control and *dFOXO* flies.

In both ANOVAs, females survived longer than males (wild-type control flies: $F(1, 1002) = 527.72$, $p < 0.0001$; 168.80 ± 2.05 vs. 122.62 ± 1.16 min; *dFOXO* flies: $F(1, 492) = 191.49$, $p < 0.0001$; 168.35 ± 3.57 vs. 100.62 ± 4.03 min). The gravity effect (Fig. 1) was not significant in control wild-type flies ($F < 1$; 1, 3 and 5 g flies: 143.54 ± 2.55, 145.82 ± 2.66, 147.78 ± 2.37 min) and significant in *dFOXO* flies ($F(2, 492) = 10.03$, $p < 0.0001$; 1, 3 and 5 g flies: 123.68 ± 5.22, 129.00 ± 5.24, 150.79 ± 5.34 min), flies surviving slightly longer when the gravity level increased, mainly at 5 g. In both wild-type and *dFOXO* flies the sex by gravity level interaction was not significant (Fs close to 2) and the genotype effect was significant (wild-type flies: $F(3, 1002) = 189.54$, $p < 0.0001$; *dFOXO* flies: $F(2, 492) = 14.98$, $p < 0.0001$), as well as the interaction between sex and genotype (wild-type flies: $F(3, 1002) = 11.56$, $p < 0.0001$; *dFOXO* flies: $F(2, 492) = 30.04$, $p < 0.0001$). The interactions of the gravity and genotype factors ($Fs < 1$) were not significant in both wild-type and *dFOXO* flies and the second-order interactions bordered significance (wild-type flies: $F(6, 1002) = 2.09$, $p = 0.0511$; *dFOXO* flies: $F(4, 492) = 2.18$, $p = 0.0706$).

To sum up, having been kept in HG, mainly at 5 g, increased the survival time at 37 °C of *dFOXO* flies, this effect being nearly not observed in wild-type flies, but HG nevertheless increased survival time of Meyzieu flies.

Nuclear translocation of dFOXO in Meyzieu flies

Figure S2 shows examples of dFOXO translocation. Males had a lower percentage of translocation than females (Fig. 2, $F(1, 6076) = 29.81$, $p < 0.0001$; percentage ± confidence interval at $p = 0.05$: 32.62 ± 1.50 vs. 40.80 ± 1.99%), and heat-shocked flies had a higher translocation than not heat-shocked ones ($F(1, 6076) = 633.62$, $p < 0.0001$; 51.38 ± 1.73 vs. 18.56 ± 1.42%). The percentage of translocation decreased in HG flies ($F(2, 6076) = 37.23$, $p < 0.0001$; 1 g: 42.79 ± 2.16, 3 g: 32.57 ± 2.55, 5 g: 32.12 ± 2.09%), but the sex by gravity interaction ($F(2, 6076) = 19.20$, $p < 0.0001$) showed that this was observed only in males (1, 3, and 5 g: 40.40 ± 2.89, 31.97 ± 2.40, 26.14 ± 2.50%) while this percentage was the lowest at 3 g in females (1, 3, and 5 g: 45.70 ± 3.24, 33.48 ± 3.49, 41.73 ± 3.56%). The gravity by heat shock interaction (F close to 2), the sex by heat shock interaction ($F < 1$), and the second-order interaction were not significant ($F < 1$).

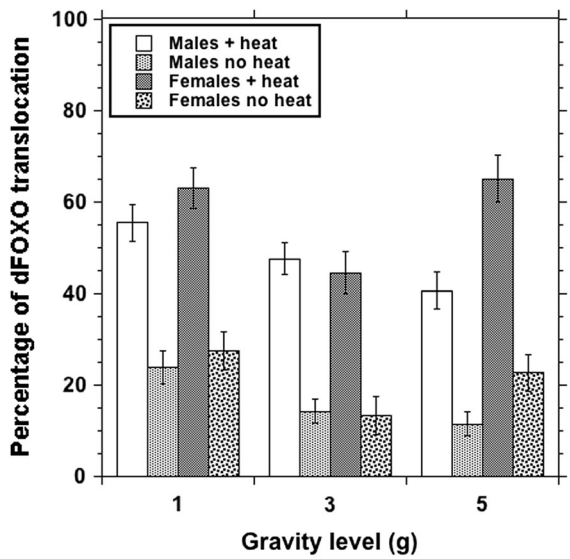

Fig. 2 Nuclear localisation of dFOXO. Percentage of nuclei (± confidence interval at p = 0.05) containing dFOXO in the visceral fat body of Meyzieu flies. Flies were kept or not in HG for 2 weeks at young age and subjected (+ heat on the figure) or not (no heat on the figure) to a 37 °C shock for 30 min at 21 days of age (5 days after the end of centrifugation) in a water-bath 30–60 min before being dissected. Twelve flies were analysed in each group and the number of observed nuclei was in the 249–769 range for each bar (33–366 nuclei with dFOXO)

To sum up, in both sexes and all gravity groups, the heat shock increased the percentage of dFOXO translocation. HG males had a lower translocation than 1 g ones, no matter they were heat-shocked or not, and a lower translocation in HG was not clearly observed in females. Therefore, because Meyzieu flies that lived in HG survive slightly longer at 37 °C than 1 g ones and translocation is lower in HG flies, it seems that translocation of dFOXO is not a critical factor to explain this better survival.

Discussion

It is now well known that mild stresses can have positive effects on resistance to severe stress in various species but also on ageing and lifespan (reviews in Le Bourg and Rattan 2008; Rattan and Le Bourg 2014). Particularly, HG (e.g. Le Bourg and Minois 1997; Minois et al. 1999), mild heat (Le Bourg et al. 2001; Hercus et al. 2003; Sørensen et al. 2007) and cold stresses (e.g. Le Bourg 2007) can increase survival time at 37 °C or to higher temperatures of *D. melanogaster* flies, and one can wonder whether these different mild stresses involve the same mechanisms to increase resistance to heat. In a previous study, we (Polesello and Le Bourg 2017) showed that a mild cold stress did not increase survival time at 37 °C of $dFOXO^{\Delta 94}$ and 21/25 *dFOXO* mutants, contrarily to what was observed in control and wild-type flies (w^{1118}, Meyzieu, mix of 21/+ and 25/+ flies), which indicates that removing dFOXO nullified the positive effect of the mild cold stress. However, because HG, cold, and heat are different stresses, one cannot conclude that other mild stresses increasing resistance to heat would share the same mechanisms as cold. The present study thus tested whether the same *dFOXO* mutants, and also $dFOXO^{\Delta 94}$/25 ones, have an increased resistance to heat after having been kept in HG for 2 weeks at young age. In sharp contrast with the effect of the mild cold stress, HG slightly increased survival time to heat of Meyzieu flies (Fig. S1a), but the effect was not significant in the other control genotypes (w^{1118}, mix of 21/+ and 25/+ flies, $dFOXO^{\Delta 94}$/+ flies). Previous studies with the wild-type strain Meyzieu (Le Bourg and Minois 1997; Minois et al. 1999; Le Bourg et al. 2002) or flies overexpressing *hsp70* and their control strain (Le Bourg et al. 2002) have shown that HG increased survival time at 37 °C but a previous unpublished study with w^{1118} only reported a not significant trend for a slightly longer survival of flies having lived in HG. HG increased resistance to heat in the *dFOXO* mutants (21/25, $dFOXO^{\Delta 94}$, $dFOXO^{\Delta 94}$/25), even if the effect in 21/25 ones was not significant. On the whole, when control genotypes and *dFOXO* mutants were analysed separately, HG increased resistance to heat of mutant flies, but this effect was not significant in control ones (Fig. 1). Because HG increases resistance to heat in *dFOXO* mutants, one can conclude that dFOXO does not mediate the effect of HG on resistance to heat, which is the opposite result to that observed with a mild cold stress (Polesello and Le Bourg 2017).

A higher translocation of the dFOXO protein after a heat shock was observed in both sexes of Meyzieu flies (Fig. 2). This higher translocation after a heat shock is similar to what has been observed in our previous study (Polesello and Le Bourg 2017) in males of the same Meyzieu strain, but also in w^{1118} and in a mix of 21/+ and 25/+ males, females being not studied in our previous study. Thus, this study confirms that heat increases dFOXO translocation in males and extends this conclusion to females.

Having lived in HG decreased translocation in males, this effect being not clearly observed in females, no matter flies were heat-shocked or not. Because Meyzieu flies survived longer to heat if they have spent 2 weeks in HG (Fig. S1a), this result confirms that a higher translocation of dFOXO does not explain the better resistance to heat of Meyzieu flies. It is in accordance with the result showing that *dFOXO* mutants better resist heat if they have lived in HG (Fig. 1): dFOXO does not explain the positive effect of HG on resistance to heat.

Otherwise, as living in HG decreased the percentage of dFOXO translocation in heat-shocked and not heat-shocked males, one could conclude that, paradoxically, living in HG is less stressful than living at 1 g (and thus that less dFOXO translocation is needed) or that flies rely on other mechanisms than dFOXO to cope with HG. Let us examine these two hypotheses.

Is living in HG less stressful than living at 1 g? Males kept in HG for 1 or 4 weeks have a lower resistance to starvation, no effect being clearly observed in females, HG does not clearly decrease resistance to desiccation or to cold in both sexes (Minois and Le Bourg 1999) and it may even slightly

increase resistance to cold (Le Bourg 2012, 2015, 2017) or not (see results with the genotypes used in this article in the Supplemental Material section). The activity of antioxidant enzymes catalase and superoxide dismutase is not modified in HG at any age (Le Bourg and Fournier 2004), in accordance with the absence of HG effect on resistance to oxidative stress (Le Bourg 2008, 2012). The lifespan of males decreases if they live for their whole life in HG but increases if they spend no more than 4 weeks in HG, while females tend to live shorter in HG (Le Bourg et al. 2000). On the whole, living in HG does not appear to be a severe stress, but it would be going too far to conclude that it is less stressful than to live at 1 g.

Do flies rely on other mechanisms than dFOXO to cope with HG? It has been shown that HSP70 could explain the better resistance to heat of flies that have lived in HG, but HSP70 are not induced if flies are not heat-shocked (Le Bourg et al. 2002). Thus, there is at least one possible mechanism explaining the better resistance to heat of flies that have lived in HG, but Donovan and Marr (2016) showed that the induction of various heat-shock proteins by the herbicide paraquat is strongly lowered in $dFOXO^{\Delta 94}$ flies. If the induction of HSP70 by heat stress would also be lowered in our *dFOXO* mutants, it would mean that the better resistance to heat of *dFOXO* mutants is not linked to the induction of heat-shock proteins. Unfortunately, no other study of the mechanisms explaining the effect of HG has been done, if we except that of antioxidant enzymes catalase and superoxide dismutase, which are not modified in HG at any age (Le Bourg and Fournier 2004). We (Polesello and Le Bourg 2017) showed that the dFOXO translocation was lower in heat-shocked males if they were previously subjected to a mild cold stress that increased survival time at 37 °C and the present results show that resistance to heat can be increased when translocation is lowered. This indicates that resistance to heat can increase even if there is a lower dFOXO translocation: it seems that flies rely on other mechanisms than dFOXO translocation to cope with heat stress. Being more speculative, one could even hypothesise that a lower dFOXO translocation is necessary to observe a better resistance to heat after a mild stress and even, because HG strongly increased heat resistance in *dFOXO* mutants but not in their control flies, that dFOXO decreases the positive effect of HG on resistance to heat. However, this would apply only to some wild-type strains, as HG increases resistance to heat of the Meyzieu strain and of flies overexpressing *hsp70* and their control strain (Le Bourg and Minois 1997; Minois et al. 1999; Le Bourg et al. 2002). Studying other wild-type strains could be useful.

Translocating dFOXO is probably not always a critical factor to explain resistance to heat, depending on the mild stress used to increase this resistance to heat. Translocation, among other mechanisms, is needed when a mild cold stress is used (the mild cold stress increases heat resistance of wild-type flies but not of *dFOXO* flies), but is useless (or even deleterious?) when HG is used (HG increases heat resistance of *dFOXO* flies). To verify this hypothesis, it would be needed to use another mild stress known to increase resistance to heat, such as mild heat stress (Le Bourg et al. 2001). It is however maybe not so surprising that *dFOXO* mutant flies can take advantage, or not, of a mild stress to resist a severe stress. On the one hand, Moskalev et al. (2011) showed that 21/25 flies did not increase their tolerance to a severe irradiation after having been subjected to a mild dose, in contrast to 25/+ flies, which is a result similar to that observed by us when using cold as a mild stress (Polesello and Le Bourg 2017). On the other hand, fasting 21/25 flies before subjecting them to a severe cold stress (Le Bourg and Massou 2015) increased their resistance to a severe cold stress, but this effect was slightly less important than in wild-type 21/+ flies. Thus, depending on the mild stress preceding a severe stress, it seems that dFOXO can be useful, useless, or even deleterious to cope with this severe stress. In addition, results could also be dependent on the severe stress, and not only on the mild stress preceding it.

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