Rapamycin induces autophagy and increases heat tolerance in *Drosophila melanogaster*

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Abstract

Autophagy is a physiological process that facilitates the recycling of intracellular cytosolic components as a response to diverse stressful conditions. By increasing the turnover of damaged structures and clearance of long-lived and larger protein aggregates, the induction of autophagy increases tolerance to abiotic stress in a range of organisms. However, the contribution of this process to heat-tolerance of insect models remains poorly studied to date. Here, we report that rapamycin exposure in *Drosophila melanogaster* induces autophagy in flies, which in turn correlates with an increase in heat tolerance and quicker recovery from heat-coma. This confirms the potentially important role of the autophagic process in heat tolerance mechanisms in this organism, opening the path to further characterization of its relationship to thermal acclimation and molecular level processes related to stress.

Keywords

Autophagy, rapamycin, heat-tolerance, insects

1. Introduction

Exploring molecular and physiological mechanisms leading to thermal acclimation and increased heat-tolerance in ectotherms has been an intense subject of research (Gonzales-Tokman *et al.,* 2020; Somero, 2020). Perhaps best studied in organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, the cellular heat-shock response (HSR) is central in increasing ectotherm's thermal limits (Lindquist, 1985; Feder and Hoffmann 1999; Richter *et al.,* 2010). The HSR entails that macromolecular damage resulting from heat-induced proteotoxic stress triggers a general response under the control of the transcription factor HSF-1 (Pirkkala *et al.,* 2001). This leads to downstream expression of heat-shock proteins, but also opens a range of molecular toolboxes to further increase an organism's resilience to stress (Richter *et al.,* 2010).

Macroautophagy (hereafter referred to as autophagy) is one of these protective tools. In essence, autophagy is a degradation pathway that facilitates the recycling of damaged proteins and larger cytosolic components in response to a multitude of stressors (Mizhushima 2007; Kroemer *et al.*,, 2010; Maruzs *et al.,* 2019). In *Drosophila melanogaster*, autophagy induction is under the control of the Target of Rapamycin (TOR) pathway (Zhang *et al.,* 2000; Wullschleger *et al.,* 2006; Maruzs *et al.,* 2019). In response to stress, or exposure to rapamycin, inhibition of TOR has been linked to the upregulation of autophagy and downregulation of the synthesis of proteins that are not involved in the stress response, at the expense of cellular proliferation (Wullschleger *et al.,* 2006). This contributes to the maintain of proteostasis, and autophagy has repeatedly been linked with enhanced tolerance to starvation, desiccation, heavy metals, toxic and oxidative damages in a variety of organisms, including insects (Moore et a., 2006; Bjedov *et al.,* 2009, Minois *et al.,* 2012; Teets and Denlinger, 2013; Mizunoe *et al.,* 2018; Zhang *et al.*, 2019; Babczyńska *et al.,* 2020, Valko *et al.*, 2021). In the specific case of heat-stress, it has been demonstrated that activation of HSF-1 induces autophagy in multiple tissues in the ectothermic nematode *Caenorhabditis elegans*, which reduces protein denaturation and increases survival (Kumsta *et al.,* 2017). Furthermore, rapamycin treatments have previously been shown to increase heat-tolerance in *D. melanogaster* (Rhode, 2021). Taken together, this provides strong circumstantial evidences for the upregulation of autophagy to be another pivotal mechanism to increase thermal tolerance in insects.

In this work, we thus report that a rapamycin enriched diet does indeed induce autophagy in the flies' gut, and confirm that this is secondary linked to an increase in heat-tolerance and recovery from heat-coma in *D. melanogaster* adults. We thus further bridge the gap between the induction of autophagy and the phenotypical heat-tolerance observed as a result of rapamycin-treatment in flies.

2. Methods

2.1 Fly Stocks and rearing

Wild *Drosophila* flies were bait-trapped in April 2019 around the Stellenbosch University campus, Western Cape, South Africa. Single female flies were isolated into 250 ml rearing flask supplied with 50ml of Bloomington cornmeal diet (Lewis, 1960) and left to lay eggs for one day. Between 2 and 5 of the first emerging adults were used for species determination following the key of Markow and Grady (2005). Stocks were kept at a density of ~100 flies per flask, at a constant 25°C and a 12:12 light-dark cycle until experiments.

2.2 Rapamycin Treatment

For larval developmental assays, rapamycin (LC Laboratories) was dissolved in molecular biology grade absolute ethanol (Sigma-Alldricht) and added to the Bloomington cornmeal diet (Lewis, 1960) at an concentrations known to induce systemic effects in flies (50 or 200µM, Bjedov *et al.,* 2009). For control (0 µM), ethanol alone was added. For heat-knockdown and recovery assays in adults, rapamycin was dissolved in ethanol and mixed with liquid food (5% yeast extract, 5% sucrose, 1% ethanol) to reach the appropriate concentration of rapamycin (50 or 200µM). For the control group (0 µM), ethanol alone was added. Blue food commercial dye (Brilliant blue FCF) was used to visually confirm feeding events in adults. 8 days old flies were left to feed on this diet for 2 days prior to experiments.

2.3 Developmental assays

Single 10 day old female flies were transferred into new vials containing the Bloomington cornmeal diet (Lewis, 1960), supplemented with 0, 50 or 200µM rapamycin, and left to lay eggs for 24h. Development of larvae in the medium at 23°C was monitored daily, and number of pupations and eclosions carefully recorded.

2.4 Heat-knockdown and recovery assays

For heat-knockdown experiments at 37°C, groups of 10 flies exposed to either 0, 50 or 200 µM rapamycin for 2 days were isolated into glass vials containing a moist cotton ball. Vials were then submerged in a programmable water bath (GD120 series, Grant Instrument Ltd) and the temperature inside the vials was monitored using 0.075 mm diameter thermocouples (Type T Thermocouple (Copper/Constantan), OMEGA Engineering, CT, USA) connected to a digital thermometer (Fluke 52-II Dual Input Digital Thermometer, WA, USA). Flies were exposed to a constant 37°C and checked every 15 minutes for loss of righting response, defined as the onset of muscle coordination when flies would fall on their back after a gentle vial shake, keep in a curled-up position, and display total absence of movements for more

than 15 consecutive minutes. Between 115 and 127 flies were used for each experimental condition to extract knockdown curves at 37°C. For heat-knockdown experiments at 41°C, because the onset of muscle coordination would occur much faster, single flies were isolated into a glass vials, exposed to 41°C, and monitored continuously. As soon as the loss of righting response occurred, each heat knocked-down fly was immediately placed back at room temperature (22°C) and monitored until recovery, defined as the time at which a fly could stand on its legs again, without stumbling (lack of coordinated movement) or falling over from external stimulation (gentle vial shaking). Between 45 and 51 flies were used to reconstruct 41°C knockdown and recovery curves as a function of time. Times to HKD at 41°C and times to recovery were finally plotted against each other and the dataset screened for potential correlations.

2.5 Dissection, staining and confocal microscopy

To visualize the induction of autophagy in *D. melanogaster*, the gut of control or rapamycin-treated flies were dissected out in 4°C chilled PBS and then stained in a 10 µM Lysotracker Red:1µg/ml Hoechst solution (LysoTracker™ Red DND-99, Hoechst 33342 Solution, Thermofisher Scientific) for 3 minutes. Each preparation was washed three times with cold PBS, fixed overnight in 4% PFA at 4°C and mounted within the next 2 hours. Imaging was performed using a Zeiss LSM 780 ELYRA P.S.1 confocal microscope using a 20x objective. In each case, the midgut region between the proventriculus and Malpighian tubules were acquired. Laser power, digital gain and optical settings were kept constant between the images. Images were then binarized using a set threshold and pixel of high values quantified with the ImageJ software 1.50 (Schneider *et al.,* 2012). The ratio of positive pixels per counting area was pooled for each individual and presented as an average made over between 7 and 13 individuals for each condition.

2.6 Statistical analysis

Kruskal-Wallis tests, Log-rank tests analyses of Kaplan-Meier survival and development curves as well as linear regressions were performed using GraphPad Prism 6.01.

3. Results

3.1 Rapamycin feeding induces autophagy in the Drosophila gut

Both larvae and adult flies were fed control or rapamycin supplemented food at 50 and 200µM, after which their guts were dissected and stained with Lysotracker prior to imaging. On average, there was a significant increase in the number of Lysotracker-positive lysosomes and autolysosomes observed in the midgut after rapamycin treatment in all life stage, indicative of autophagy induction (Fig.1 A, B). Quantification of the average number of

pixels above intensity threshold in midgut areas of flies was indicative of a dose-dependent response as a function of rapamycin concentration in food (Fig. 1C, Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons post hoc test, p<0.01).

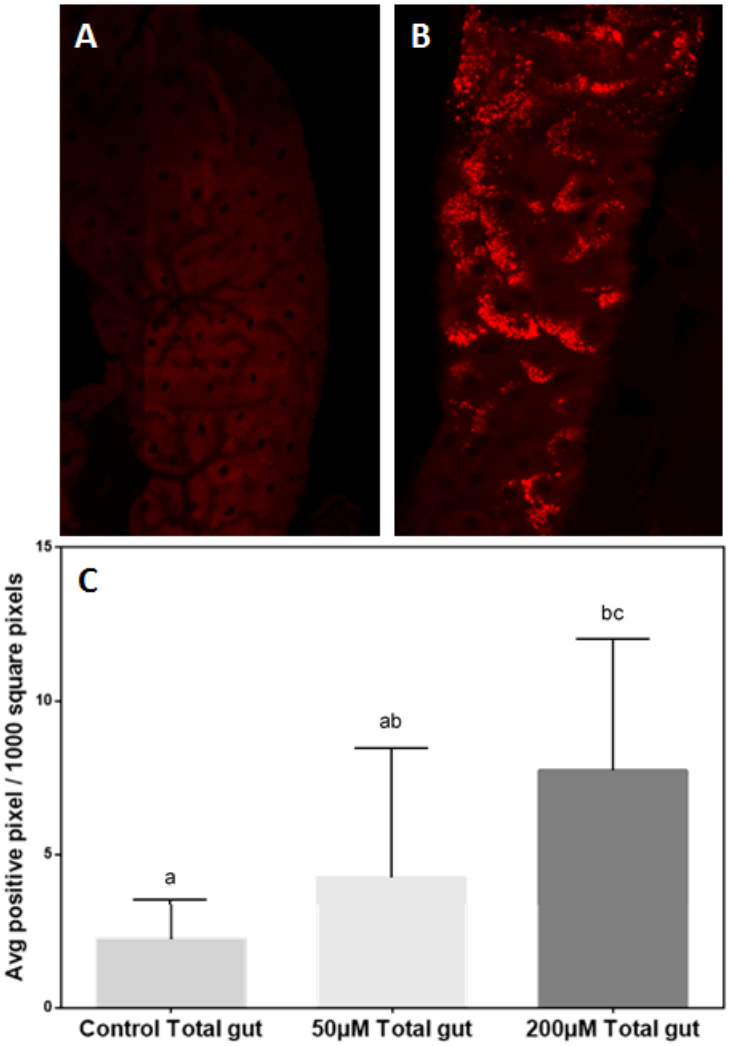

Figure 1. Representative confocal fluorescence images of adult *Drosophila* midguts stained with LysoTracker Red (scale bar is 50 µM). **A.** Midgut of control flies. **B.** Midgut of rapamycin treated flies (200 µM). **C.** Average number of pixels above intensity threshold over midgut images of 7 to 13 individuals per conditions. Different lowercase letters indicate significant differences between treatments (Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons post hoc test, P<0.01).

3.2 Rapamycin feeding delays Drosophila larval development

Delayed larval development as a result of TOR inhibition is a documented effect of rapamycin exposure in *D. melanogaster* (Zhang *et al.,* 2000). We thus performed larval growth assays in order to confirm that, in our experimental setup, a rapamycin-enriched diet would indeed induces the expected systemic effects associated with TOR inhibition, in addition to autophagy induction. Larvae were left to develop in Bloomington cornmeal diet (Lewis, 1960) supplemented with either 0 (control), 50 or 200 µM of Rapamycin. The number of pupations and imagoes hatching was recorded as a function time at 23°C. 50 µM

rapamycin exposure significantly delayed the onset of first larval pupations by about 4 days and hatching by 6 days (Fig.2; Log-ranked test, p<0.001). Survival from first instar to imago was estimated at 98% in control conditions (N=453) and dropped to 36% with 50 µM supplemented rapamycin food (N=129). No pupation event was observed at 200 µM (all larvae remained either in second or third instars until mortality reached 100% after 20 days).

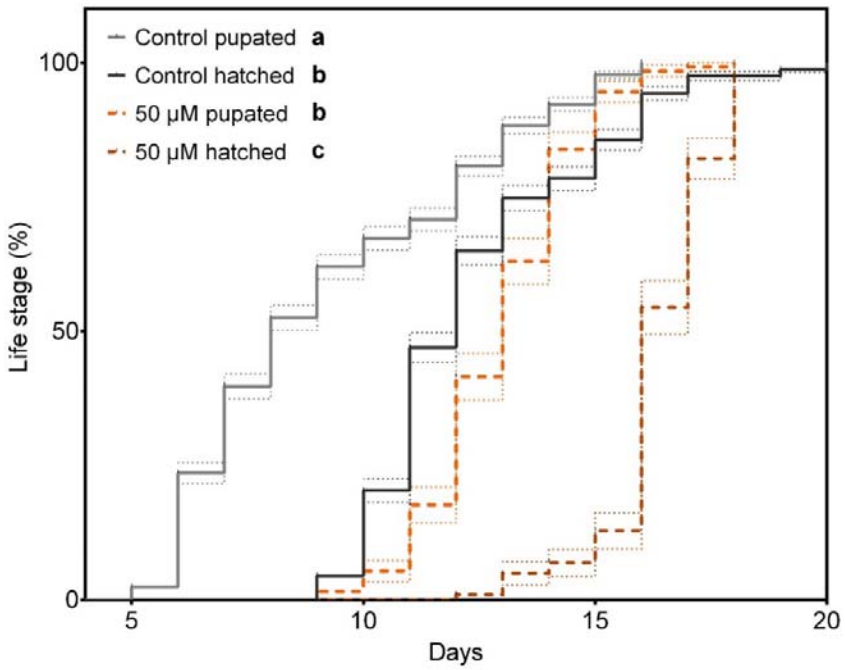

Figure 2. Number of *Drosophila melanogaster* pupation and hatching events as a function of time at 23°C, for larvae fed either control or a 50 µM rapamycin supplemented diet. Rapamycin exposure delays both the onset of pupation and hatching by several days. Different lowercase letters indicate significant differences between treatments (Log-rank tests, P<0.001).

3.3 Rapamycin treatment delays time to knockdown in flies at 37°C

Eight-day old adult flies were fed a rapamycin-enriched diet at either 0 (control), 50 or 200 µM for two days and then subsequently exposed to a static 37°C stress to induce a time-dependent heat knockdown. The number of knocked down flies as a function of time was recorded every 15 minutes (Fig. 3). Rapamycin exposure delayed the time for flies to enter knockdown (Fig. 3, Log-ranked test, $p<0.001$). 50 µM rapamycin exposure in food was already sufficient to induce a plateau response.

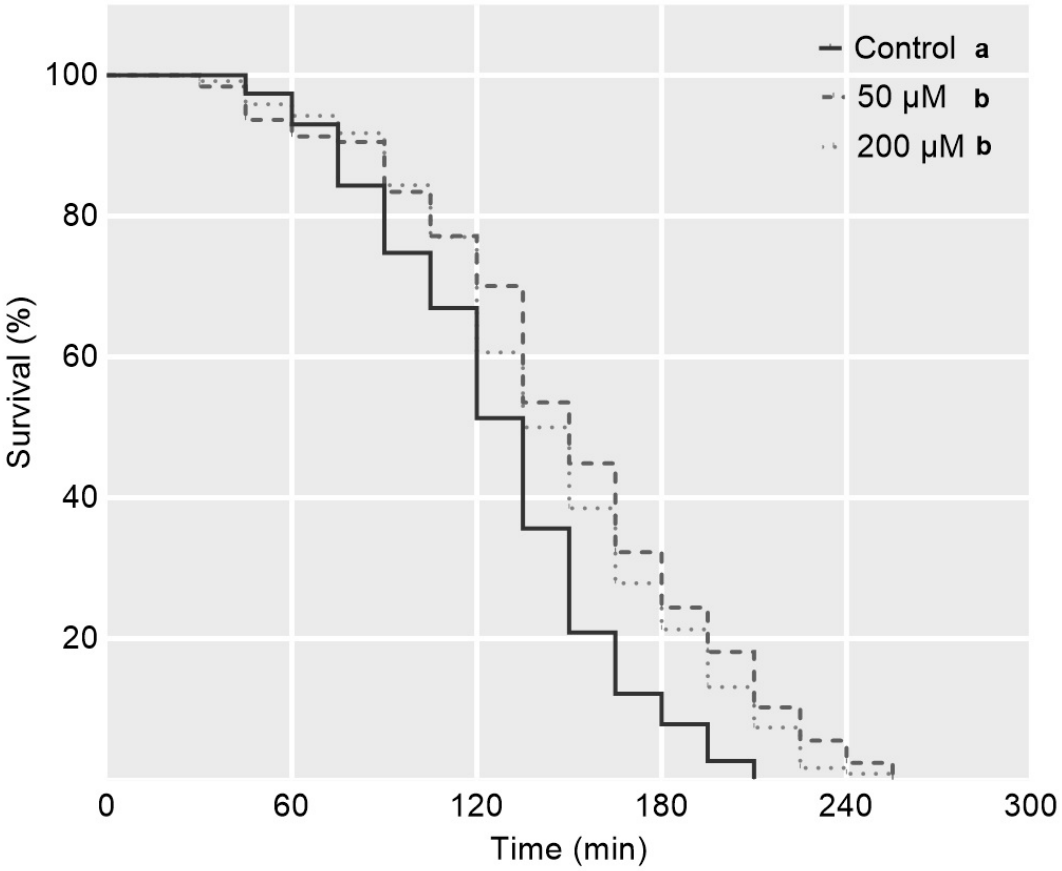

Figure 3. Survival of *Drosophila melanogaster* flies that were exposed to a rapamycin-enriched diet as a function of time at 37°C. Rapamycin exposure delayed time-to knockdown in flies, and 50 µM exposure was sufficient to induce a plateau response. Different lowercase letters indicate significant differences between treatments (Log-rank tests, $P<0.001$).

3.4. Rapamycin treatment decreases time to recovery post knockdown

Eight-day old adult flies were fed a rapamycin-enriched diet at either 0 (control), 50 or 200 µM for two days and subsequently exposed to a constant 41°C stress to induce a fast heat-knockdown. Flies were monitored continuously and put back at room temperature (23°C) for recovery after the loss of muscle coordination. The impact of rapamycin exposure on fast knockdown and its linked recoveries are presented in Fig. 4. For knockdowns at 41°C, no effect of rapamycin exposure in delaying time to knockdown was observed (Fig. 4A). However, rapamycin exposure reduced the time to recovery post knockdown in flies (Fig. 4B, Log-ranked test, $p<0.001$). Finally, for each individual fly, time to knockdown at 41°C was plotted against its time to recovery, and significant regressions were screened both as a function of rapamycin exposure (Fig. 5A) and on the global dataset (Fig. 5B; Table 1). Only a weak correlation between time to knockdown and recovery was found for flies fed with 50 µM rapamycin ($R^2=0.22$, $p <0.001$, Fig. 5A) and on the global dataset ($R^2=0.05$, $p<0.01$; Fig. 5B). No significant correlations were recorded for other conditions (Table 1).

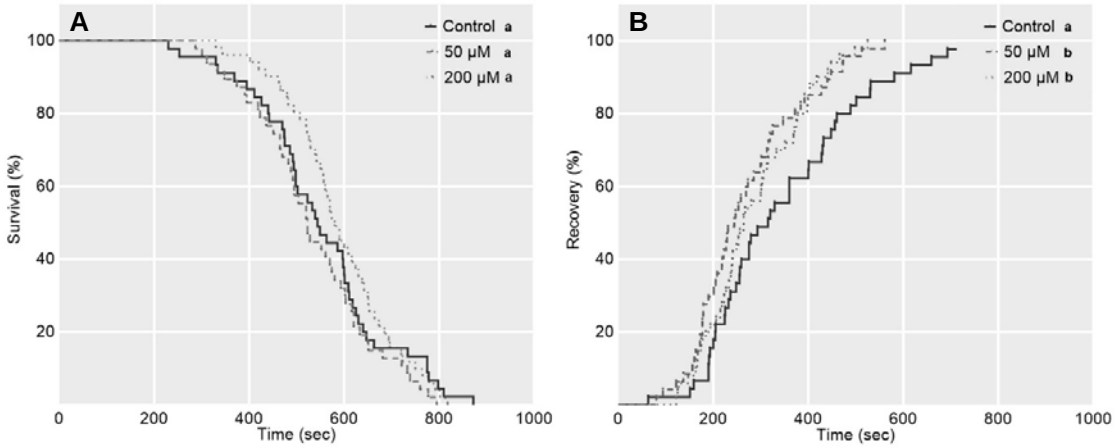

Figure 4. Heat knockdown and recovery curves of *D. melanogaster* flies that were exposed to a rapamycin-enriched diet as a function of time at 41°C. **Fig. 4A.** Rapamycin exposure did not delay time to heat knockdown in flies at 41°C**. Fig. 4B.** Rapamycin exposure decreased time to recovery from heat knockdown of flies. Different lowercase letters indicate significant differences between treatments (Log-rank tests, $P<0.001$).

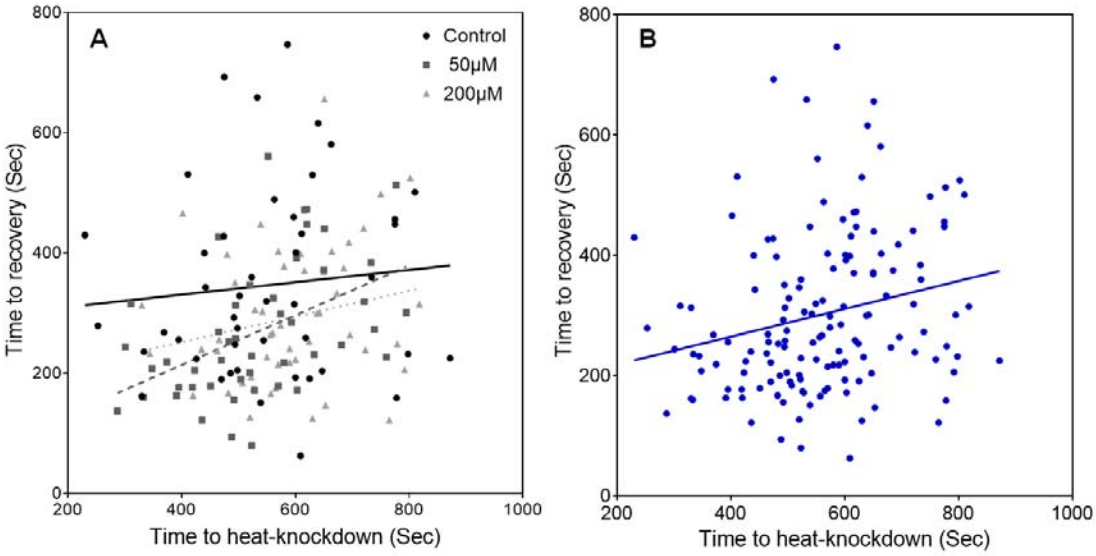

Figure 5. Time to recovery as a function of time to heat-knockdown at 41°C in flies**. Fig. 5A**: Linear regression for individuals pooled as a function of treatment. No significant correlation was observed, with exception of a weak linear relationship in 50 µM rapamycin fed flies (R^2=0.22, p<0.001; Table 1). **Fig.5B**: regression on the global dataset, a minor but statistically significant linear correlation was present (R^2= 0.05, *p*<0.01; Table 1).

Table 1. Details of the linear regressions calculated for time to recovery as a function of time to heat-knockdown at 41°C in individual flies. A weak but statistically significant relationship was observed for flies fed rapamycin at 50 µM and on the global dataset.

Condition	Equation	R^2	*p* value
Rapamycin 0 µM (Control)	Y = 0,1028*X + 289,9	0.008	0.54
Rapamycin 50 µM	Y = 0,4141*X + 48,21	0.220	$9*10^{-4}$
Rapamycin 200 µM	Y = 0,2135*X + 166,5	0.044	0.13
Global dataset	Y = 0,2315*X + 172,3	0.051	$6.3*10^{-3}$

4. Discussion

The inhibition of TOR as a result of rapamycin exposure in *D. melanogaster* is a well-documented process (Lamming, 2016). First, in this species, rapamycin feeding increases the number of visible lysosomes and/or autolysosomes in the midgut stained with Lysotracker (Bjedov *et al.,* 2009), which was also confirmed in our results (Fig. 1). This indicates intracellular absorption of the drug through feeding, and a probable local inhibition of TOR, triggering an increase of autophagic flux in exposed tissues. Second, rapamycin is known to reduce the downstream phosphorylation of p70 S6 kinase involved in ribosome biogenesis, and disrupts signaling of cell nutrient availability (Zhang *et al.,* 2000). These effects likely account for an important part of the delayed *Drosophila* cellular growth and proliferation observed after rapamycin exposure (Zhang *et al.,* 2000), which we also witnessed in our experiments through hindered larval development (Fig. 2). Taken together with the increased autolysosome formation in the gut, this indicates that rapamycin feeding does cause the expected systemic effects in link with TOR inhibition in our experimental system, and inducing autophagy at least locally in the gut.

Overall, autophagy's role in protecting from abiotic stressors such a desiccation, toxics, or direct oxidative damages in ectotherms has been highlighted numerous times (Moore *et al.,* 2006, Bjedov *et al.,* 2009; Minois *et al.,* 2012; Teets and Denlinger, 2013; Mizunoe *et al.,* 2018; Zhang *et al.,* 2019; Babczyńska *et al.,* 2020, Valko et al., 2021). In the specific case of thermal stress, further correlations between autophagy genes upregulation and cold acclimation have been made (Gerken *et al.,* 2015; Des Marteaux *et al.,* 2017; Tachibana *et al.,* 2019). However, a causal link between autophagy induction, heat-acclimation and increased upper heat tolerance had never been proven until more recently in *Caenorhabditis elegans* (Kumsta *et al.,* 2017). Our results first confirm that rapamycin treatment induces autophagy in flies (Fig.1), ant that this correlates with an increase in heat-tolerance (Fig. 3) also recently documented in *D. melanogaster* (Rohde *et al.,* 2021). Second, we also show that 50 µM is already sufficient to reach a plateau response under a 37°C stress exposure. Such delayed heat-knockdown was not observed in experiments conducted at 41°C, which likely underpins that knockdowns occurred too fast in this condition for us to pick a statistically significant response (Fig. 4A). Third, we were able to detect a benefit of rapamycin exposure in reducing time to recovery post heat-knockdown (Fig. 4B). This indicates that autophagy induction could be involved in both basal heat-hardiness and in heat-stress recovery. And finally, we found little correlation by plotting individual flies' times for heat-knockdown at 41°C with their times to recovery (Fig. 5). This means that the heat-hardier flies (that took longer to lose their righting response), were not necessarily the ones to recover faster from heat knockdown, independently of treatments. This also suggest heat-tolerance and stress-recovery to be at least based on partly different set of mechanisms

(Willot *et al.*, 2021). Overall, our results thus further bridge the link between TOR inhibition, autophagy induction, and heat-tolerance in *D. melanogaster*.

Autophagy is known to articulate with other molecular level processes related to the stress response (Kroemer *et al.*, 2010). Example of these mechanisms studies in insects have been for example the heat-shock proteins induction (Feder and Hoffmann, 1999,) the antioxidant response (Lalouette *et al.,* 2011, Valko, 2021), up-regulation of proteasomal degradation pathways (Colinet *et al.,* 2007; Kirstensen *et al.,* 2016), modification of cellular membranes (Hazel, 1995; Overgaard *et al.,* 2005; Koštál *et al.,* 2013) and the mitochondrial function (Colinet *et al.,* 2017; Havird *et al.,* 2019; Chung and Schultze 2020). At the molecular level, TOR inhibition and autophagy upregulation as a result of rapamycin exposure is likely to promote protein homeostasis and clearance of toxic protein aggregates (Kumsta *et al.,* 2017). In turn, enhanced protein homeostasis would explain the increased heat-tolerance and decreased recovery time from heat-knockdown we observed after exposing flies to rapamycin. However, the onset of heat-coma in *D. melanogaster* correlates with neural dysfunction (Jorgensen *et al.*, 2020) and in this context, the mechanisms by which increased autophagy could prove beneficial to withstand heat-stress from a neurological perspective requires more careful exploration. In *C. elegans*, the full benefit of heat-hardening was indeed reliant, but only partially, on the expression of genes involved in several steps of the autophagy process (Kumsta *et al.,* 2017). It would thus be particularly interesting to appreciate the exact contribution of autophagy induction in the context of thermal acclimation adopting a gene-based approach in the brain. Finally, exploring the relationship between heat-stress and modulation of chaperone-mediated autophagy (CMA) would be another relevant aspect to investigate. This mechanism, mediated by the molecular chaperone hsc70, directly guides proteins to be degraded to the lysosome (Cuervo and Wong, 2013). CMA thus represents a distinct protein degradation pathway from the proteasome, and could be proven of importance to increase protein turn-over and the context of heat-stress tolerance.

In conclusion, our results show that rapamycin exposure induces autophagy in *D. melanogaster*, which correlates with an increase in heat-tolerance and speed of recovery post-knockdown in adults. This highlights the induction of autophagy as potentially key mechanism to increase heat-tolerance in insects. A deeper exploration of autophagy's role in relation to other stress responsive pathways remains however necessary, and could provide important perspectives on the broader integrated cellular stress-response to temperatures in ectotherms.

Authors' contribution

Quentin Willot: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Visualization. **Andre du Toit:** Methodology, Investigation. **Ben Loos:** Conceptualization, Methodology, Resources, Writing - review & editing. **John S. Terblanche:** Conceptualization, Resources, Writing - review & editing, Funding acquisition.

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Competing interests

We declare we have no competing interests.

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