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Beta-guanidinopropionic acid does not extend *Drosophila* lifespan

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ABSTRACT

Activation of AMP activated protein kinase (AMPK) signaling has been demonstrated to extend lifespan and improve healthspan across multiple species. This suggests pharmaceutical approaches to increase AMPK hold the potential to modify the aging process and promote healthy aging. Beta-guanidinopropionic acid (GPA) is a naturally occurring metabolite structurally similar to creatine. GPA is capable of activating AMPK signaling in mammalian models via competitive inhibition of cytosolic creatine kinase. A previous report suggested that dietary GPA supplementation increased lifespan in *Drosophila* through its effect on AMPK signaling and regulation of autophagy. However, studies in *Caenorhabditis* have found no beneficial effect of this compound on worm lifespan and that GPA may actually diminish lifespan in at least one *Caenorhabditis* species. To confirm previous reports of increased longevity in *Drosophila*, we tested a wide range of GPA concentrations on lifespan and healthspan in both male and female W^{1118} flies. We report here that GPA does not extend lifespan in *Drosophila* as previously reported. Moreover, high doses of GPA are detrimental to *Drosophila* lifespan and stress resistance in male flies. These results suggest the lack of a robust effect of GPA on *Drosophila* lifespan and highlight the importance of replication studies within the field of aging.

1. Introduction

AMP activated protein kinase (AMPK) signaling has been shown to play a critical role in regulation of lifespan across multiple species [1–3]. Thus, pharmaceutical activators of AMPK may potentially be used to enhance health and longevity. Among these, beta-guanidinopropionic acid (GPA) is a naturally occurring *in vivo* AMPK activator that has been shown to act as a competitive inhibitor of the mammalian cytosolic creatine kinase (cCK) [4,5] resulting in depletion of phospho-creatine levels, increased cellular AMP:ATP ratio, and ultimately increased activation of AMPK [6–8]. Previous studies have shown GPA is capable of improving exercise tolerance, enhancing glucose uptake, and increasing expression of mitochondrial oxidative enzymes [9] in rodents suggesting that it may hold potential for improving both lifespan and healthspan.

A previous study by Yang et al. suggested that dietary supplementation of GPA at either 900 mM or 2700 mM extended lifespan in *Drosophila* in an AMPK-dependent manner [10]. This study also reported that GPA improved stress resistance, reduced glycolysis, and extended lifespan beyond that of dietary restriction. While these results support GPA as a pro-longevity intervention, a more recent study from the *Caenorhabditis* Intervention Testing Program (CITP) found that GPA delivered between 0.1 μM and 10 mM failed to increase lifespan across three Caenorhabditis species and across multiple sites of testing. Moreover, 300 mM GPA, a dose 3–9 times lower than the range previously reported as beneficial in *Drosophila*, significantly *reduced* lifespan in *C. briggsae* in these studies [11].

These contrasting studies raise significant questions regarding the conserved nature of GPA mediated alterations to lifespan and healthspan including whether the effects of GPA are specific to species, strain, or laboratory conditions. Here, we attempted to repeat the previously reported experiments with GPA in *Drosophila* using similar, though not identical, husbandry and treatment conditions. Moreover, we attempt to determine whether GPA mediates similar effects on lifespan and healthspan. We report that GPA does not improve *Drosophila* lifespan and may be detrimental to both longevity and physiological function at higher doses. This report serves to highlight the importance of testing longevity interventions at multiple sites to better understand the robustness of reported outcomes.

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2. Methods

2.1. Animal models

For experiments with 900 mM GPA W^{1118} flies were provided as a gift from Dr. Andrew Pickering at UTHSCSA and were maintained at room temperature (approx. 25 °C) under ambient lighting (approx. 12-hr light cycle). This particular strain had been inbred in this local laboratory for several generations. Following initial experiments in this local strain, we chose to use a standardized fly strain that could be obtained from a central source to make it easier for others in the future to replicate our experiments. For experiments with 100 nM–100 mM GPA, W^{1118} flies were sourced from Bloomington Drosophila Stock Center (3605) and maintained at 25 °C under a 12-hr light cycle. All flies were maintained on Nutri-fly BF (Genesee Scientific).

2.2. Drug treatment

Beta-guanidinopropionic acid (GPA) was obtained from Henan Tianfu Chemical Co., LTD Zhengzhou China. Food containing 100 nM–100 mM GPA was prepared by dissolving GPA in water and adding it to food (<50 °C) at the time of preparation. Flies were collected at eclosion (day 0) and allowed to mate for 3 days before treatment, n = 20/vial. 900 mM food was prepared by adding a slurry of GPA in water to food (<50 °C) and stirring to completely dissolve. Flies were collected 24–48 h post eclosion and placed on treatment, n = 19–30/vial. All flies were transferred to fresh vials of food containing drug or control 3 times weekly.

2.3. HPLC assessment of GPA purity

GPA originating from Henan Tianfu Chemical Co. was dissolved in water at two different concentrations (0.1 and 1.0 μg/ml) and compared with a GPA standard purchased from Sigma-Aldrich (St. Louis, MO; CAT#: G6878, LOT#: BCBZ3820) dissolved in water at the same two concentrations. Peak areas of four samples of GPA solution from Henan Tianfu Chemical Co. were compared with a single sample of the analytical standard using a previously described HPLC/MS/MS method [12]. The GPA obtained from Henan Tianfu Chemical Co. was found to have similar purity to the analytical standard from Sigma-Aldrich (median: 102.5%, intraquartile range (IQR): 97.2-106.7%).

2.4. Spontaneous activity

Spontaneous activity was assessed after 50 days of treatment using Trikinetics Drosophila Activity Monitor (Trikinetics LAM10). Activity was recorded in vials of 8–11 flies (n = 3/grp) maintained at 25 °C under a 12-hr light cycle.

2.5. Stress testing

Flies were pretreated with 100 nM–100 mM, GPA (or not for control) for 7 days prior to testing as described above. H_2O_2 testing was carried out in vials containing filter paper wetted with 10% sucrose and 15% H_2O_2 in PBS. Starvation was assessed on 1.5% agar in PBS. Flies were maintained at room temperature for periodic monitoring.

2.6. Statistical analysis

Survival was analyzed by log-rank test. Activity was analyzed by One-way ANOVA. All analyses were performed using GraphPad Prism 8 (GraphPad).

3. Results

To evaluate the effect of GPA on *Drosophila* lifespan we first attempted to reproduce the results of Yang et al. The highest concentration reported in this previous study, 2700 mM GPA (354.1 g/L), was several times greater than the solubility limit in either water or prepared food and we were thus unable include it in our study. In our initial screen utilizing a particularly long lived W^{1118} line, we found 900 mM GPA (118 g/L) significantly *decreased* both male (~54%) and female (~63%) W^{1118} lifespan (Fig. 1A–B). These results are inconsistent with the study by Yang et al., which reported a small (~10%) increase in male and female lifespan with 900 mM and 2700 mM GPA. Given the apparent toxicity of high concentration GPA under our testing conditions, we instead asked if lower dosages may be beneficial. Using 100 nM, 100 μM, or 100 mM GPA, we found no significant effect on male or female fly lifespan (Fig. 2A–B). Notably, these results are consistent with the work by Yang et al., which reported no effect of GPA on lifespan at 300 mM in *Drosophila*. Moreover, the CITP reported no effect of GPA on lifespan in *Caenorhabditis* at lower doses in the range of 0.1 μM–10 mM and detrimental effects at higher (300 mM) dosing in worms.

Yang et al. also reported that pretreatment with GPA improved resistance to starvation and H_2O_2 exposure in male and female *Drosophila*. As 900 mM GPA increased mortality in our experiments, we instead pretreated 3 day old male and female flies for 7 days with 100 nM-100 mM GPA prior to testing. In females, 100 μM GPA improved survival following exposure to 15% H_2O_2. However, neither 100 nM nor 100 mM GPA produced any effect. In contrast, male flies treated with either 100 nM or 100 mM GPA exhibit reduced H_2O_2 survival (Fig. 3A). 100 mM GPA also reduced male starvation resistance but had no effect

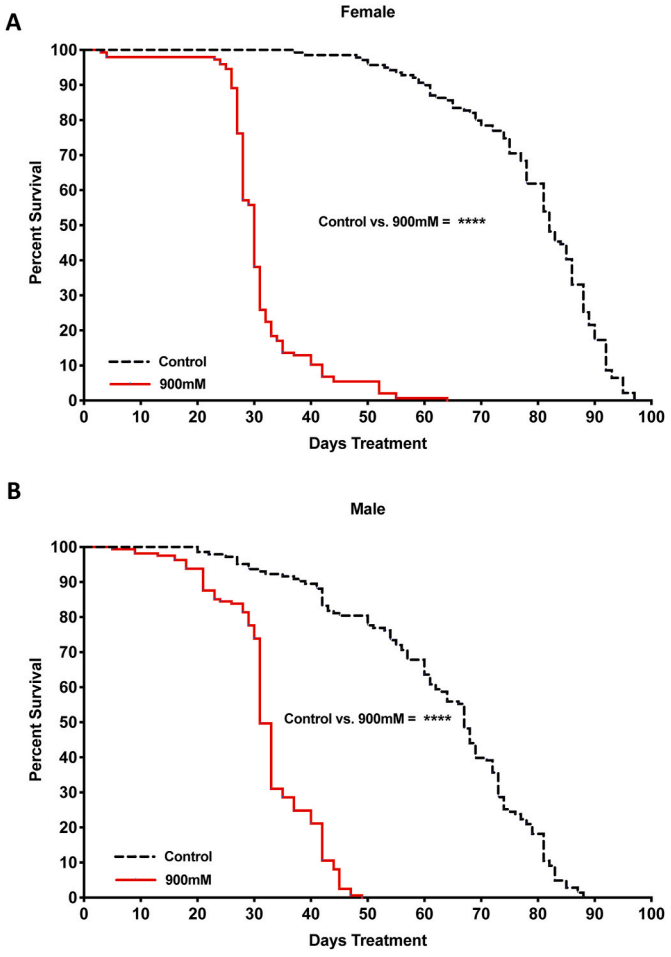

Fig. 1. Survival of long lived female (A) and male (B) W^{1118} flies fed Control, or 900 mM beta-guanidinopropionic acid. Curves compared individually to sex-specific control by log-rank test. Survival of ♀ (n = 139–147/grp; log-rank vs ctrl: p=<0.0001) and ♂ (n = 143–161/grp; log-rank vs ctrl: p=<0.0001).

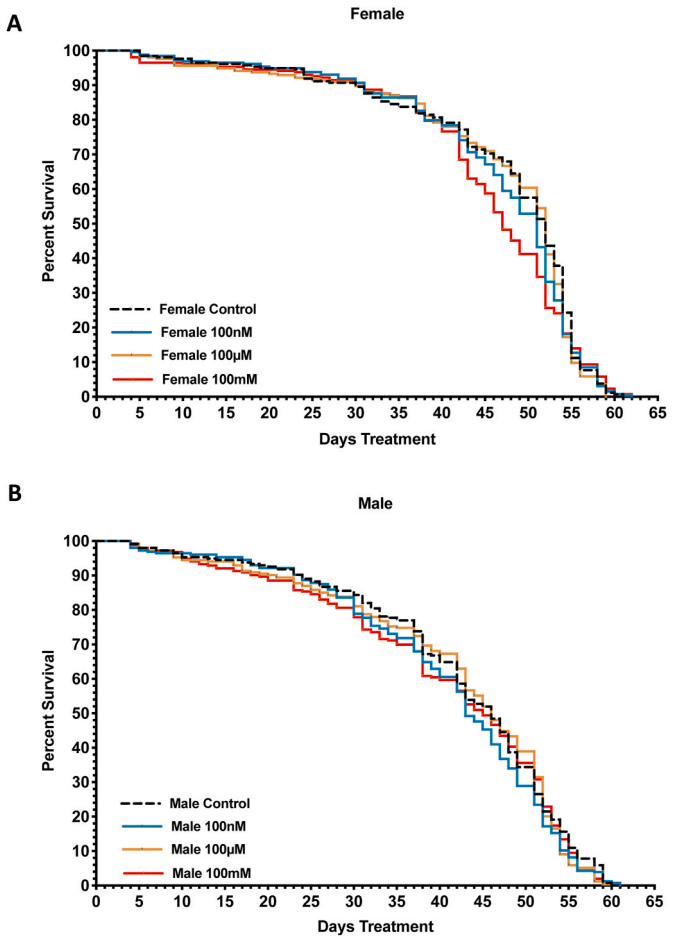

Fig. 2. Survival of female (A) and male (B) W^{1118} flies fed Control, 100 nM, 100 μM, or 100 mM beta-guanidinopropionic acid. Curves compared individually to sex-specific control by log-rank test. Survival of ♀ (n = 255–259/grp; log-rank vs ctrl - 100 nM: p = 0.1904; 100 μM: p = 0.3005; 100 mM: p = 0.0547) and ♂(n = 253–256/grp; log-rank vs ctrl - 100 nM: p = 0.1496; 100 μM: p = 0.4031; 100 mM: p = 0.3213).

on females. (Fig. 3B). While our outcomes are dissimilar to those of Yang et al., it must be noted that we used a shorter (7 day) pretreatment period than that previously reported (30 day). It is conceivable that longer treatment under energetic stress may further stimulate stress response processes, including mitochondrial biogenesis, and that may be beneficial for overall stress resistance. We do note that the small, but significant, differences in stress resistance might be within the normal variability for repeated experiments despite being statistically significant. Moreover, the small beneficial effect of GPA on female H_2O_2 resistance is only apparent at a single dose, hinting at low robustness of this outcome. Perhaps more importantly though, even a lack of effect supports a dissimilarity in results to those reported by Yang et al.

Age-related loss of activity in *Drosophila* has been well established, and AMPK signaling has been reported to preserve climbing ability in aging [2,13]. Therefore, as an additional marker of functional aging, we measured voluntary activity at 50 days of treatment. Flies were monitored for 24 h using a *Drosophila* Activity Monitor, n = 3 vials/grp, 8–11 flies/vial (Fig. 3C).

Neither male nor female total daily activity, light cycle activity, or dark cycle activity were altered by GPA in the sample sizes that we assessed for this parameter (Fig. 3D–F). Along with the other data we present here on functional effects in *Drosophila,* these data suggest limited effect on activity of GPA treatment.

4. Discussion

Similar to Yang et al., our results demonstrate that GPA delivered in food in the range of 100 nM–100 mM is insufficient to extend lifespan or modify measures of functional aging in *Drosophila.* Moreover, we observed that 900 mM GPA, a dose previously suggested to increase lifespan, actually *shortened* median lifespan by 54% in males and 64% in females. In some ways, these results mirror recent reports published by the CITP [11] in which they found GPA had no effect on Caenorhabditis lifespan between 0.1 μM and 10 mM, but 300 mM GPA significantly shortened the lifespan of C. briggsae. A similar pattern was observed in our experiments examining stress resistance with 100 mM GPA leading to reductions in both H_2O_2 and starvation resistance in male flies. Taken together, these results seem to suggest that dietary GPA supplementation in the 100 mM range and above may lead to significant toxicity in both flies and worms.

Previous reports have suggested that GPA mediates its effects on lifespan via inhibition of cCK and activation of AMPK. While AMPK is conserved across species, neither *Drosophila* nor *Caenorhabditis* possess cCK, but instead possesses a functionally similar arginine kinase (Argk) [14,15] which may not be able to utilize GPA as a substrate preventing it from acting as a competitive inhibitor [16]. As hypothesized by the CITP [11], this difference in phosphagen kinases between species likely explains the lack of lifespan alteration observed in this study and theirs.

It is unclear why we were unable to replicate the findings regarding GPA and *Drosophila* lifespan previously reported by Yang et al. While the source of GPA used in these studies differs, our HPLC analyses shows our GPA source to be of high purity and similar to that sold by Sigma (median: 102.5%, intraquartile range (IQR): 97.2-106.7%; see methods). While we believe this supports the idea that both sources should be relatively equivalent in their effect, it is possible that other components, such as trace levels of containments, might drive the differences in our studies. However, as we discussed above the highest concentrations of GPA reportedly used in the Yang et al. study where much higher than we could solubilize in water or food during initial testing. While we were able to prepare food with 900 mM GPA, our results suggest this was extremely detrimental to male and female *Drosophila.* It is hard to reconcile these differences and it is unclear whether this is due to direct toxicity or indirect effects such as reduced palatability of the food and impairment of food intake. While we used the Yang et al. study as a guide for our own work, there are several potential differences between our studies that are not directly reported but might drive our disparate findings. For example, there are clear differences between our groups in the ability to solubilize GPA at high concentrations for food preparation. Differences in the homogeneity of GPA, or drug precipitation during food prep, might alter the distribution of this compound in the food which might then contribute to different outcomes between studies. Moreover, the process of food preparation and the potential degradation of GPA might drive these discrepancies in ways in which haven't yet been determined. Other potential external factors including food viscosity, relative humidity, final pH, microbial growth or microbiome makeup might also be plausible effectors of these differences that should likely be addressed. All these differences have been associated with changes in fly behavior and add to the complicated issue of replicating studies, particularly lifespan studies in different laboratories. While clarifying these differences is beyond the scope of this study, they all are potential important next steps to address in resolving the issue of what is the effect of GPA on lifespan.

The work reported here highlights the challenges in replication of studies particularly of those addressing lifespan. However, by highlighting the differences in our studies we also highlight the lack of a robust effect of this intervention on *Drosophila* lifespan. Our results are however similar to the effect of GPA on lifespan reported in Caenorhabditis in that we found no effect on lifespan except *reduction* of lifespan at higher doses. It would be of interest to take a more standardized and thorough approach to test the effects of GPA, and other prolongevity

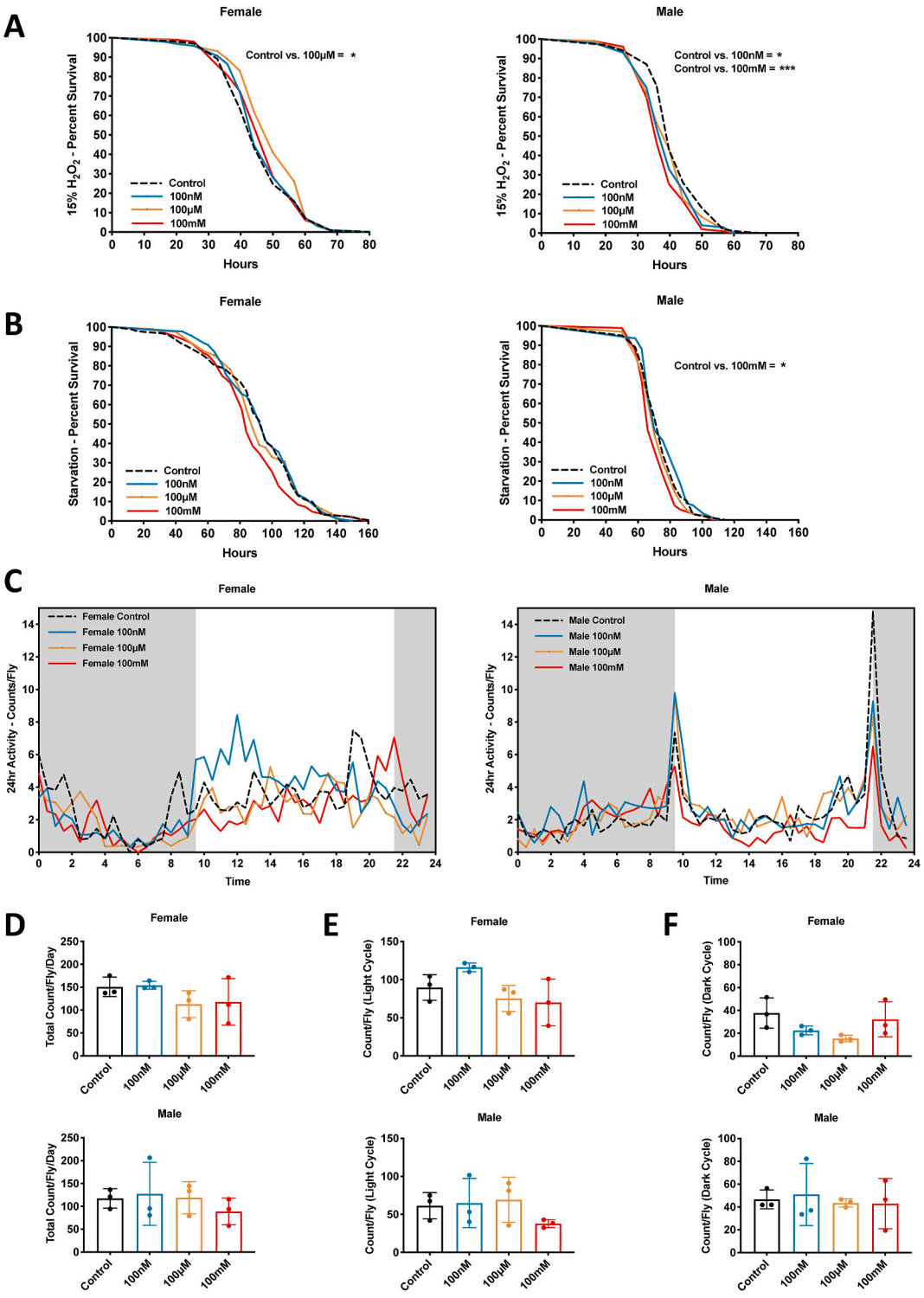

Fig. 3. 15% H_2O_2 survival (A) for ♀(n = 96–101/grp; log-rank vs ctrl −100nM: p = 0.6146; 100 μM: p = 0.0212; 100 mM: p = 0.4844) and ♂(n = 97–103/grp; log-rank vs ctrl −100 nM: p = 0.0278; 100 μM: p = 0.1069; 100 mM: p = 0.0001). Starvation resistance (B) for ♀(n = 83–91/grp; log-rank vs ctrl – 100 nM: p = 0.9224; 100 μM: p = 0.7495; 100 mM: p = 0.0783) and ♂(n = 93–101/grp; log-rank vs ctrl – 100 nM: p = 0.3766; 100 μM: p = 0.3400; 100 mM: p = 0.0305). 24-hour activity (C) with quantification of total counts (D), and individual light cycle (E), and dark cycle (F) activity for W^{1118} flies fed Control, 100 nM, 100 μM, or 100 mM beta-guanidinopropionic acid. Measurements for (C–F) made at day 50 of treatment. N = 3 vials/grp, 8–11 flies/vial. Total counts normalized to number of flies per vial. Comparisons by one-way ANOVA. Data displayed as mean ± SD.

compounds, across multiple species in an effort to identify robust interventions capable of modifying longevity.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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JDD formulated the study. JDD, KMT, & BCG collected the data. JDD & BCG analyzed the results. JDD created the figures and drafted the manuscript. JDD & ABS edited the manuscript. JDD, KMT, BCG & ABS reviewed and approved the manuscript. GPA concentrations were measured by Greg Friesenhahn of the Biological Psychiatry Analytic Laboratory at UT Health San Antonio and the Analytical Pharmacology and Drug Evaluation Core of the San Antonio Nathan Shock Center.

References

[1] M.B. Wierman, N. Maqani, E. Strickler, M. Li, J.S. Smith, Caloric restriction extends yeast chronological life span by optimizing the Snf1 (AMPK) signaling pathway, Mol. Cell Biol. 37 (2017), https://doi.org/10.1128/MCB.00562-16.

[2] M. Ulgherait, A. Rana, M. Rera, J. Graniel, D.W. Walker, AMPK modulates tissue and organismal aging in a non-cell-autonomous manner, Cell Rep. 8 (2014) 1767–1780, https://doi.org/10.1016/j.celrep.2014.08.006.

[3] J. Apfeld, G. O'Connor, T. McDonagh, P.S. DiStefano, R. Curtis, The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans, Genes Dev. 18 (2004) 3004–3009, https://doi.org/10.1101/gad.1255404.

[4] R. Chevli, C.D. Fitch, β-Guanidinopropionate and phosphorylated β-guanidinopropionate as substrates for creatine kinase, Biochem. Med. 21 (1979) 162–167, https://doi.org/10.1016/0006-2944(79)90068-1.

[5] E.A. Boehm, G.K. Radda, H. Tomlin, J.F. Clark, The utilisation of creatine and its analogues by cytosolic and mitochondrial creatine kinase, Biochim. Biophys. Acta Bioenerg. 1274 (1996) 119–128, https://doi.org/10.1016/0005-2728(96)00018-7.

[6] D.B. Williams, L.N. Sutherland, M.R. Bomhof, S.A. Basaraba, A.B. Thrush, D. J. Dyck, C.J. Field, D.C. Wright, Muscle-specific differences in the response of mitochondrial proteins to beta-GPA feeding: an evaluation of potential mechanisms, Am. J. Physiol. Endocrinol. Metab. 296 (2009) E1400–E1408, https://doi.org/10.1152/ajpendo.90913.2008.

[7] E.A. Shoubridge, R.A. Challiss, D.J. Hayes, G.K. Radda, Biochemical adaptation in the skeletal muscle of rats depleted of creatine with the substrate analogue beta-guanidinopropionic acid, Biochem. J. 232 (1985) 125–131, https://doi.org/10.1042/bj2320125.

[8] C.D. Fitch, M. Jellinek, E.J. Mueller, Experimental depletion of creatine and phosphocreatine from skeletal muscle, J. Biol. Chem. 249 (1974) 1060–1063.

[9] I. Oudman, J.F. Clark, L.M. Brewster, The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review, PloS One 8 (2013), https://doi.org/10.1371/journal.pone.0052879 e52879.

[10] S. Yang, L.H. Long, D. Li, J.K. Zhang, S. Jin, F. Wang, J.G. Chen, beta-Guanidinopropionic acid extends the lifespan of Drosophila melanogaster via an AMP-activated protein kinase-dependent increase in autophagy, Aging Cell 14 (2015) 1024–1033, https://doi.org/10.1111/acel.12371.

[11] A.L. Coleman-Hulbert, E. Johnson, C.A. Sedore, S.A. Banse, M. Guo, M. Driscoll, G. J. Lithgow, P.C. Phillips, Caenorhabditis Intervention Testing Program: the creatine analog beta-guanidinopropionic acid does not extend lifespan in nematodes, Micro Publ. Biol. 2020 (2020), https://doi.org/10.17912/micropub.biology.000207.

[12] J.D. Dorigatti, K.M. Thyne, B.C. Ginsburg, A.B. Salmon, Beta-guanidinopropionic acid has age-specific effects on markers of health and function in mice, Geroscience (2021), https://doi.org/10.1007/s11357-021-00372-8.

[13] Y. Sun, J. Yolitz, C. Wang, E. Spangler, M. Zhan, S. Zou, Aging studies in Drosophila melanogaster, Methods Mol. Biol. 1048 (2013) 77–93, https://doi.org/10.1007/978-1-62703-556-9_7.

[14] T. Wallimann, H.M. Eppenberger, Properties of arginine kinase from Drosophila melanogaster, Eur. J. Biochem. 38 (1973) 180–184, https://doi.org/10.1111/j.1432-1033.1973.tb03048.x.

[15] D. Fraga, M. Aryal, J.E. Hall, E. Rae, M. Snider, Characterization of the arginine kinase isoforms in Caenorhabditis elegans, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 187 (2015) 85–101, https://doi.org/10.1016/j.cbpb.2015.05.002.

[16] Y. Robin, C. Klotz, T. Nguyen Van, Unspecific arginine kinase of molecular weight 150000, Eur. J. Biochem. 21 (1971) 170–178, https://doi.org/10.1111/j.1432-1033.1971.tb01453.x.