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Natural Variation In Recovery From A Short Period Of Anoxia Due To A cGMP-Dependent Protein Kinase in *Drosophila melanogaster*

Abstract

cGMP-dependent protein kinases (PKG) have been shown to play an important role in resistance to abiotic stressors such as high temperatures or oxygen deprivation in *Drosophila melanogaster*. In *Drosophila*, the *foraging* gene encodes a PKG; natural variants for this gene exist, which differ in the level of expression of PKG: rovers (for^R allele) which express high PKG levels, and sitters (for^S allele) which express lower PKG levels. This project explores the differences in recovery from short periods of anoxia between natural variants (focusing on for^{S2}, flies with a sitter gene in a rover background), as well as mutants with insertions in the *foraging* gene and RNA_i recombinants that show a reduced PKG expression. The parameters measured were time to recovery and level of activity after anoxia. The results showed lower activity after anoxia in sitters than in rovers, reflecting a worse recovery from the anoxic coma in flies with lower PKG levels.

Introduction

Animals have to cope with abiotic stress constantly. Rapid changes in environmental conditions, such as floods, oxygen deprivation or fast increases or decreases in temperature affect vital functions of organisms and challenge their ability to survive. Big problems often have big solutions, though, and it is fascinating to see how well some organisms have adapted to these abrupt and usually unpredictable changes. In this thesis anoxia will be the focus, and mechanisms evolved in *Drosophila melanogaster* to deal with it.

Drosophila melanogaster is an interesting model system to study mechanisms for anoxia tolerance. The fruit fly has been demonstrated to survive to periods with very low levels of O_2 (Haddad, 2006), being able to resist for as long as 6 hours in a level of zero oxygen. At very low oxygen levels, neural failure occurs leading to spreading depression (SD) and eventual synaptic transmission failure that results in a state of coma; because this failure happens before permanent neural damage, flies recover after the stress conditions have passed.

This is why spreading depression is believed to have a neuroprotective role; a temporary shutdown of neural activity protects the whole system from permanent damage caused by total energy depletion. SD is characterized by a dramatic surge of extracellular K^+ and temporary inactivity in the CNS (Rodgers et al., 2010), in a process analogous to the cortical SD (CSD) in mammals (Van Harreveld and Khattab, 1967, Aitken et al., 1991), including humans (Gorji et al., 2001). CSD is considered to be the source of the aura that accompanies migraine, as well as of stroke and seizures (Hadjikhani et al., 2001). Understanding tolerance of animals to anoxia and the processes leading to CSD could help understand and mitigate these pathologies.

Even if SD has been identified in many organisms, the exact molecular mechanisms that underlie such process remain poorly known. *Drosophila melanogaster* is an appropriate model to discover molecules and pathways involved in SD, not only because of its high resistance to anoxia but also because its low cost, small size and short life cycle make it an attractive organism to use in several drug applications. Furthermore, its well-studied genetics and wide availability of genetic tools make it an easy engineering target to study the contribution of different genes in SD.

This thesis explores the role of a cGMP-dependent protein kinase (PKG) in the recovery from anoxic coma in *Drosophila melanogaster*. PKGs are serine/threonine kinases found in a wide range of eukaryotic organisms (Feil et al. 2005), and they are key components of signaling cascades behind complex processes such as the regulation of foraging behavior and energy homeostasis. A known downstream target of PKG is protein phosphatase 2-A (PP2A); PKG is known to phosphorylate and activate PP2A, which in turn dephosphorylates K^+ channels increasing their conductance (Dawson-Scully et al., 2010), a pathway of special interest coming to the onset of an anoxic coma through SD.

There are natural variants of *Drosophila melanogaster* with different expression levels of PKG. Variations in the *foraging* gene give rise to two alleles: sitter (30% of natural populations) and rover (70% of natural populations). Sitter flies (for^S) display lower PKG levels compared to rover flies (for^R). In the past, the two phenotypes were characterized by their different foraging behaviors, both in larvae and adult flies: rover flies show a more active behavior, moving between food patches and rarely staying in the same patch for a long time; sitter flies, however, show a less active behavior and tend to remain longer in the food source (Sokolowski 1980). These differences in behavior are very likely to be driven by several effects of *for*; for example, high PKG levels are known to provide higher sucrose responsiveness and ability to adapt to repeated sucrose stimulation, a trait that has been associated to a successful foraging behavior in *Drosophila* and honeybees (Scheiner et al., 2004*a*, Scheiner et al., 2004*b*), as well as a greater ability to associate certain odors to the presence of food (Kaun et al., 2007).

However, as stated above, not only food related traits are affected by the foraging gene; rover and sitter flies also show different levels of resistance to abiotic stressors such as high temperatures or anoxia. Recent studies in Drosophila larvae have shown that higher levels of PKG are associated to a decreased thermotolerance, as lower PKG levels or pharmacological inhibition of either PKG or PP2A resulted in a longer time until mouth hook movements failed and synaptic transmission decayed under high temperatures (Dawson-Scully et al., 2007). The

results in adults were not as pronounced, but still sitters showed a higher thermotolerance as long as the range of temperatures was narrow, without reaching extreme temperatures (Chen et al., 2011).

Several research studies on anoxia tolerance have come up with similar results; flies with lower PKG levels resisted longer before falling in an anoxic coma (Dawson-Scully et al., 2010; Caplan et al., 2013). However, this cannot be directly linked to a higher hypoxia tolerance; in fact, for^{R} larvae and adult flies show a significantly higher survival to prolonged anoxia than for^{S} or for^{S2} (mutant flies with a sitter allele in a rover background) individuals (Wingrove and O'Farrel, 1999; Dawson-Scully et al. 2010). To add new insights to the current knowledge about the role of PKG in anoxia tolerance, this study searches for differences in the recovery from a short period of 0% oxygen between natural PKG variants, as well as flies with mutated foraging genes resulting in lower PKG levels, by analyzing time to recover and level of activity after anoxia. We hypothesize that flies with lower PKG levels will recover worse than those with higher levels, as previous studies with long lasting anoxic conditions suggested.

Materials and methods

1. Fly stocks

Adult flies of 4-7 days old were used in all the experiments. All the flies were raised in the laboratory for at least three generations for acclimation before using them for the experiments. The flies were raised on a 12 hour light/dark cycle, and fed with a standard sucrose-yeast-agar medium, and checked on a regular basis to avoid crowding or contamination. The average temperature of the room where they were kept was 22^{o}C. Three phases of experiments were conducted, and different fly strains were used in each of them.

1.1. Foraging variants

Rover (for^{R}), sitter (for^{S}) and sitter in a rover background (for^{S2}) flies were used for the first experiments. These flies were donated by the Sokolowski lab and raised in ours for three generations for acclimation.

1.2. PKG mutants

In the second phase of experiments, flies that carried insertions in the PKG gene were used. The stocks used were: 20645, 38112, 37776, 10382 and 14019 (FlyBase IDs: FBst0020645, FBst0038112, FBst0037776, FBst0010382, FBst0014019), where each of the strains carried a different insertion in different positions of the gene. There was no functional annotation for those strains, so all of them were tested initially to observe the effect of each insertion on locomotion, and decide which were eligible to be used in further locomotive assays.

1.3. Recombined flies

3 different types of RNA_i- carrying flies (35158, 31592 and 31698) were crossed with a strain carrying Elav-Gal4; recombination of these two strains produced flies with reduced PKG mRNA expression in the nervous tissue. Elav is a gene that is only expressed in neurons and is required for the correct expression of GAL4. Therefore, in the recombined lines GAL4 is only expressed in neurons, where it acts as an enhancer for the RNAi gene, which in turn reduces the expression of the *foraging* gene in those cells. To create the recombinant flies, virgin females of the RNA_i strains were isolated (0 to 5 hours old) and crossed with Elav-Gal4 young males (one week old). Control crossings were made using w1118 virgin females and males of the rest of strains. The crossings made were the following:

1: 35158 x Elav-Gal4

2: 31592 x Elav-Gal4

3: 31698 x Elav-Gal4

4: w1118 x Elav-Gal4

5: w1118 x 35158

6: w1118 x 31592

7: w1118 x 31698

The progeny was raised in the lab and used in the experiments.

Canton-S (CS) and w1118 flies were used as a reference in the other assays. Those flies have been raised in our lab for many generations and their performance in anoxic conditions has been thoroughly assessed.

2. Behavioral experiments

2.1. Isolation of males

Only male flies were used in the experiments, and thus they were isolated prior to the experiments. To facilitate isolation, flies aged 0-3 days were knocked down by exposing them to anoxia, and 4 days were allowed to pass before performing the assays to ensure full recovery.

2.2. Locomotive assays

Locomotive assays were performed in a 31 x 16 plaque with 8 x 16 cells of ½ inch diameter and 2 mm of depth, and one fly was placed in each of them; the depth of the arenas would not allow them to fly, so they were restricted to walking. The whole setup is shown in supplemental figure 1.

The assays were recorded for 71 min: 5 min of acclimation that were not included in the statistical analysis, 5 min of control for locomotive activity before anoxia, 30 seconds of 100%

N_2 exposure (anoxia) and 60 minutes of recovery. An air pump provided homogeneous room air supply in the steps that did not involve anoxia. The speed was 8-10 L/min for the N_2 pump and 2L/min for the air pump.

Control assays of the same duration but with no anoxia were performed too.

3. Data analysis

The videos were analyzed using the softwares open computer vision 2.0 (OpenCV2.0) and Microsoft Visual C++ 2008 Express. First, the background was learnt and, after, the coordinates of fly position over time were calculated. The video recorded 15 frames per second, which were analyzed in steps of 3. The raw data provided by the fly tracking softwares were transferred to an Excel template where the coordinates were translated to distance walked. Recovery was assessed by calculation criteria; flies that moved at least 0.3 cm/s, 10 times per min were considered to be recovered. From the data analysis, Time to Recovery (TR), distance walked before and after anoxia and distance walked the last ten minutes of recording were measured. The distance walked was used to estimate the level of recovery after anoxia. Flies that did not recover during the whole assay were taken into the analysis assuming the maximum time to recovery (3600 s); however, flies that remained inactive during the whole time of analysis (even before anoxia) were removed from the data analysis.

4. Statistical analysis

The program GraphPad Prism 5 was used to do a statistical analysis of the results. ANOVA was performed to compare all the strains as well as groups of them, and t-tests were used to make paired comparisons between strains. A significance level of p=0.05 was set. In ANOVA, Kruskal-Wallis and Dunn's tests were performed. In t-tests, a Welch's correction was applied.

Results

1. Foraging variants

1.1. Time to recovery

No big difference was observed in Time to Recovery between strains. A significant difference between the TR of CS and For^S was observed, though; For^S showed a higher Time to Recovery. There was no difference between For^{S2} and CS flies, making the difference between For^{S2} and For^S significant too; and there was no significant difference between For^R and For^{S2}. A slight difference between the TR between For^R and For^S flies could be found in the T-test comparing those two strains.

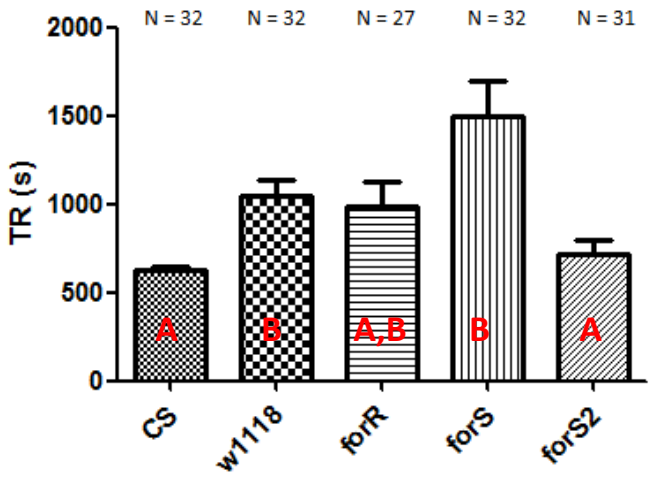

Figure 1. Differences between foraging variants and control flies in Time to Recovery. A significantly higher Time to Recovery was observed in for^{S} flies compared to the control. Values (mean ± SEM, from left to right): 627.59 ±22.05, 1045.5 ± 94.98, 988.52 ±134.0, 1497.06 ± 197.3, 713.37± 86.08. Error bars represent standard error.

1.2. Path covered in 60min after anoxia

A highly significant difference between for^{S2} and the rest of the strains could be observed regarding the 60min path after anoxia: the path of for^{S2} was much smaller than that of the other strains. It differed significantly from CS as well as from for^{R}, and also from for^{S} but with a bit lower level of significance. for^{S} and for^{R} did not differ significantly from wild-type flies (CS), although their variances were significantly different (while the variance within CS flies is rather small, for^{R} and for^{S} flies show high divergence from fly to fly).

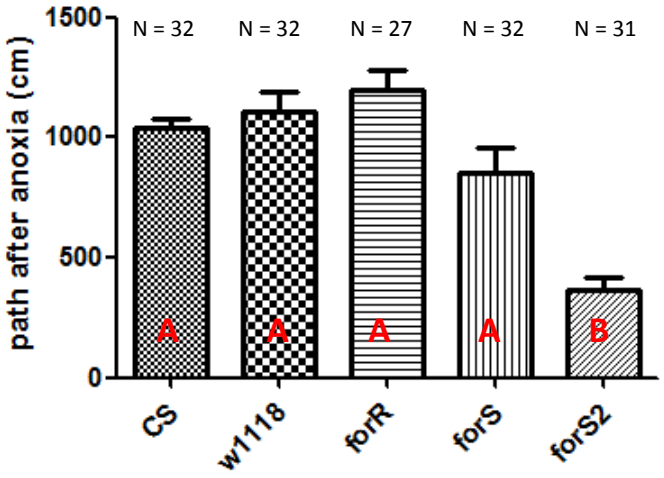

Figure 2. Differences between foraging variants and control flies in path length after anoxia. A significantly shorter path length is covered by for^{S2} flies compared to the rest of the strains. Values (mean ± SEM, from left to right): 1041 ± 37.89, 1109 ± 77.50, 1201 ± 81.09, 849.9 ± 108.5, 362.3 ± 52.63. Error bars represent standard error.

1.3. Path covered in the last 10min

The reason for doing this analysis is that the representations of the 60min path after anoxia showed an interesting pattern, in which For^{S2} flies appeared to have a fast recovery (as it was shown in the statistical analysis of TR, in which the TR of CS and For^{S2} flies didn't differ significantly), but after being active for some time their activity ceased in most of the cases, staying almost still for the last minutes (see supplemental figure 2). This behavior results in a decreased 60min path after anoxia, but the whole path after recovery is considered in that analysis, including the first active minutes. Thus, an analysis of the last minutes alone could provide interesting information about differences in recovery from anoxia between the different strains.

The results of this analysis are similar to those reported with the 60min path, but the differences between strains are enhanced. For^{S2} had a highly significantly shorter path length than all the other strains, including For^{S}. The difference between For^{R} and For^{S} remained significant but small. In addition, while in the 60min path analysis For^{R} and w1118 flies appeared to have a longer path lengths than CS flies but the p value was not small enough, in this analysis that difference became significant, showing a significantly longer path length in For^{R} flies than in CS flies. The path length for the For^{R} flies was more similar to that of w1118, which did not differ significantly.

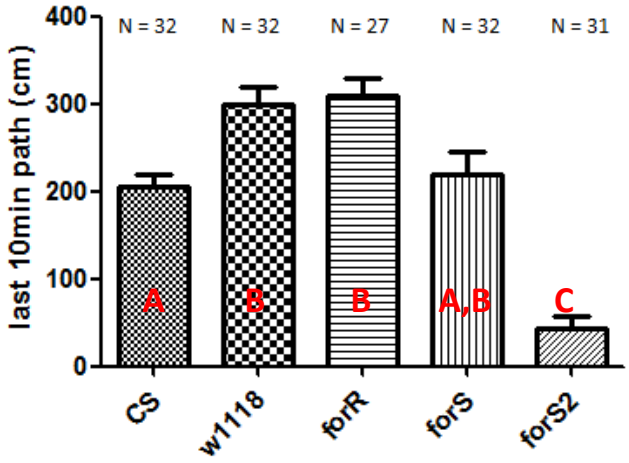

Figure 3. Differences between foraging variants and control flies in path length for the last 10min of recording. The difference between for^{S2} flies and the rest of flies is more abrupt when the statistical analysis is restricted to the last 10 min of the assay. Values (mean ± SEM, from left to right): 205.5 ± 13.28, 299.3 ± 19.93, 310.1 ± 18.72, 218.8 ± 27.22, 43.27 ± 13.30. Error bars represent standard error.

1.4. Differences in path length before anoxia

A statistical analysis was made for the first 5 minutes of recording to assess the differences in activity between strains before being exposed to anoxia. The comparison of levels of activity before and after anoxia is shown in figure 4. Even if the path length of for^{S2} flies during the first 5 minutes is significantly shorter than that of for^{R} and for^{S} flies, this difference is not as big as in the distance walked after anoxia. Furthermore, the activity of for^{S2} flies before anoxia is higher than that of CS flies, while it is drastically lower after anoxia.

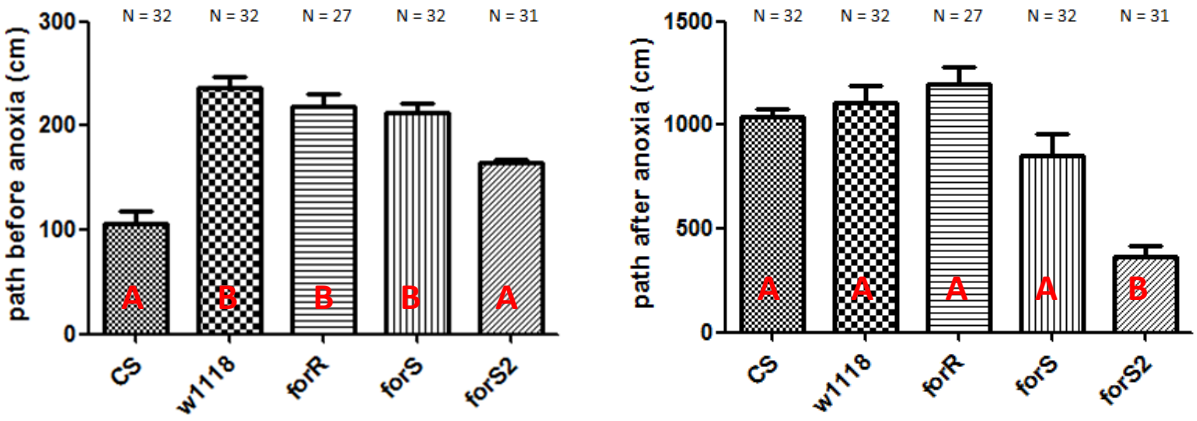

Figure 4. Comparison between path lengths before and after anoxia. While the distance walked by for^{S2} flies is longer than in CS flies before anoxia, it is highly reduced after anoxia, where for^{S2} flies show significantly lower activity than the rest of flies.

1.5. Control assay without anoxia exposure

A control assay was performed with the same strains, recording for the same period of time but without anoxia exposure, to prove that the final decrease in activity observed in for^{S2} flies was a result of the anoxic coma and not a spontaneous decrease in activity unrelated to anoxia exposure. In those assays, the flies maintained the initial activity over the whole recording time. The sample size was 56 for each strain.

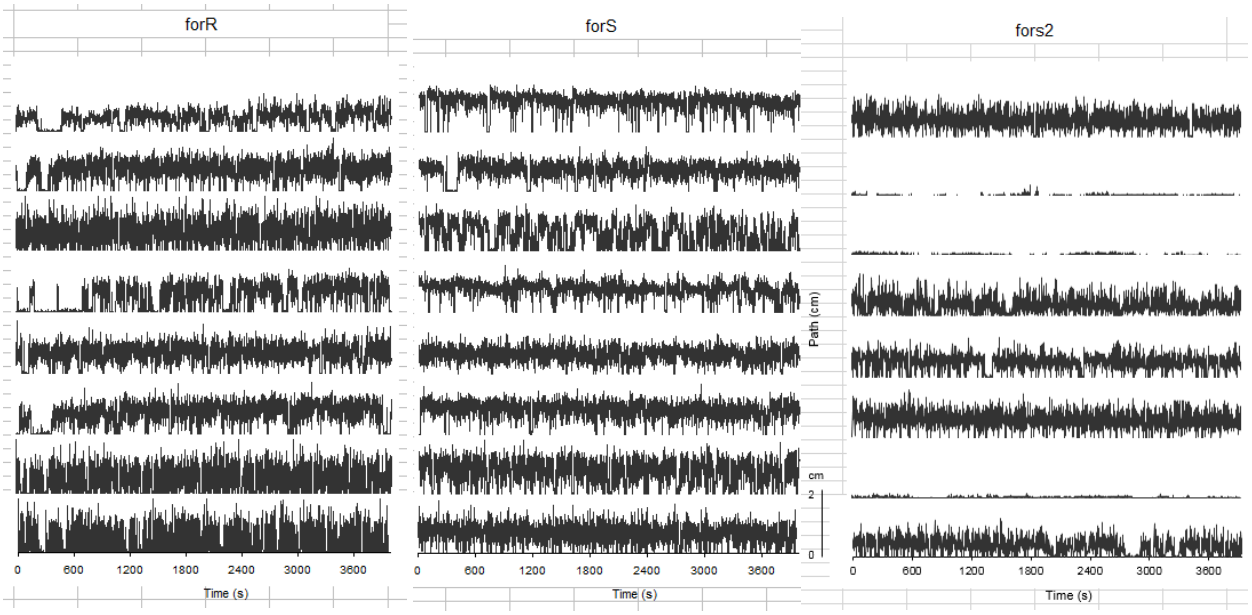

Figure 5. Results of path length over time in the assays run with no anoxia. Each row represents the activity one fly. Some of the for^{S2} flies remain inactive during the whole assay (3/8); however, those that were active from the beginning keep the same activity levels during the whole recording time. Some for^{R} and for^{S} flies also remained inactive during the whole assay (0.375/8 and 2.4/8, consecutively).

2. PKG mutants

The results for PKG mutants were not consistent. Many flies were very inactive from the beginning of the assay, and thus had to be removed from the data analysis as no information about recovery could be extracted from them. The results also varied significantly between assays. In the first two assays, all the strains remained highly inactive compared to w1118. Only the strains 14079 and 38112 showed a higher level of activity even though it was still significantly lower than that of w1118 (both before and after anoxia). In the subsequent two assays, though, this trend changed, and it could be seen that in the strains 37776 and 10382, which were very inactive in the first assays, there was a big increase in activity after recovery from anoxia (even though these flies showed very low or no activity before anoxia) (see supplemental figure 3). The differences between assays might be due to changes in room conditions (e.g. temperature variation); however, the reason of the increase of activity after anoxia remains elusive. Nevertheless, even if the results on activity after anoxia are not consistent between assays, all of them show that the flies have a highly reduced activity before anoxia. Sample sizes were: 10382: 48; 14079: 57; 20645: 47; 37776: 38; 38112: 52; w1118: 56.

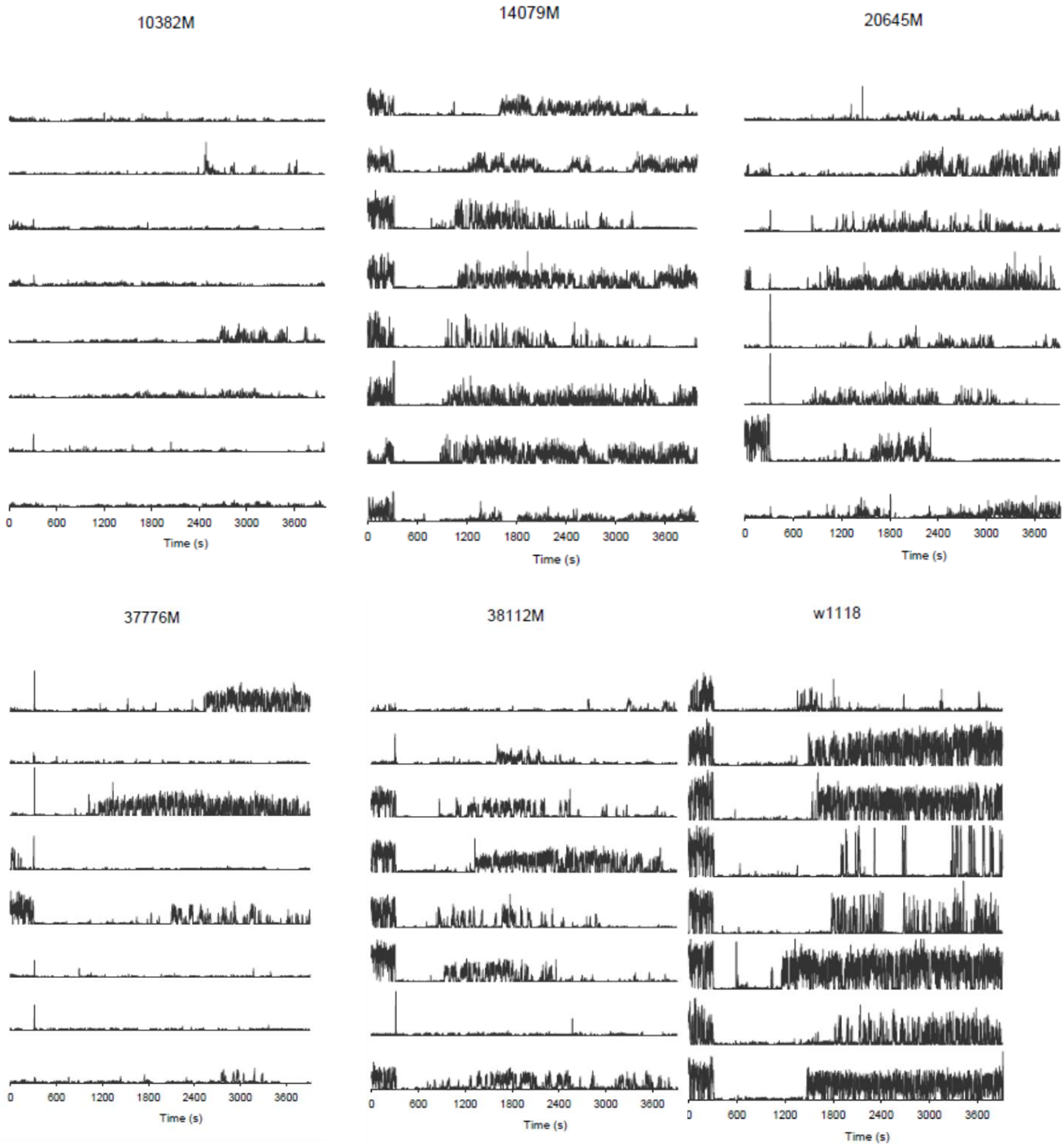

Figure 6. Results of first assay with PKG mutants. All the strains showed very low activity even before anoxia, except 14079 and 38112; however, these are still less active than the w1118 control flies. Each row represents the activity of one fly.

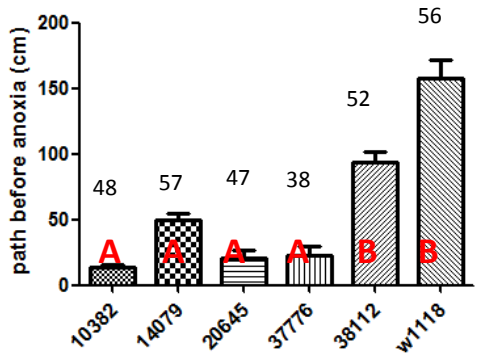

Figure 7. Differences between mutants and control flies in path length for the 5 min prior to anoxia exposure. Values (mean ± SEM, from left to right): 13.85 ± 1.826; 49.26 ± 5.689; 20.88 ± 5.656; 22.47 ± 6.835; 93.20 ± 8.603; 157.3 ± 14.18.

3. RNA_i recombinants

Assays were performed with 3 strains of recombinant flies (3 RNAi strains x UAS-GAL4) as well as 4 controls (crossings of w1118 females with males of all the other strains. The recombinant flies did not show any significant differences with the controls either in path after anoxia or time to recover (even if in TR the control of Elav x w1118 differed slightly from other controls). However, these flies showed lower activity levels before anoxia.

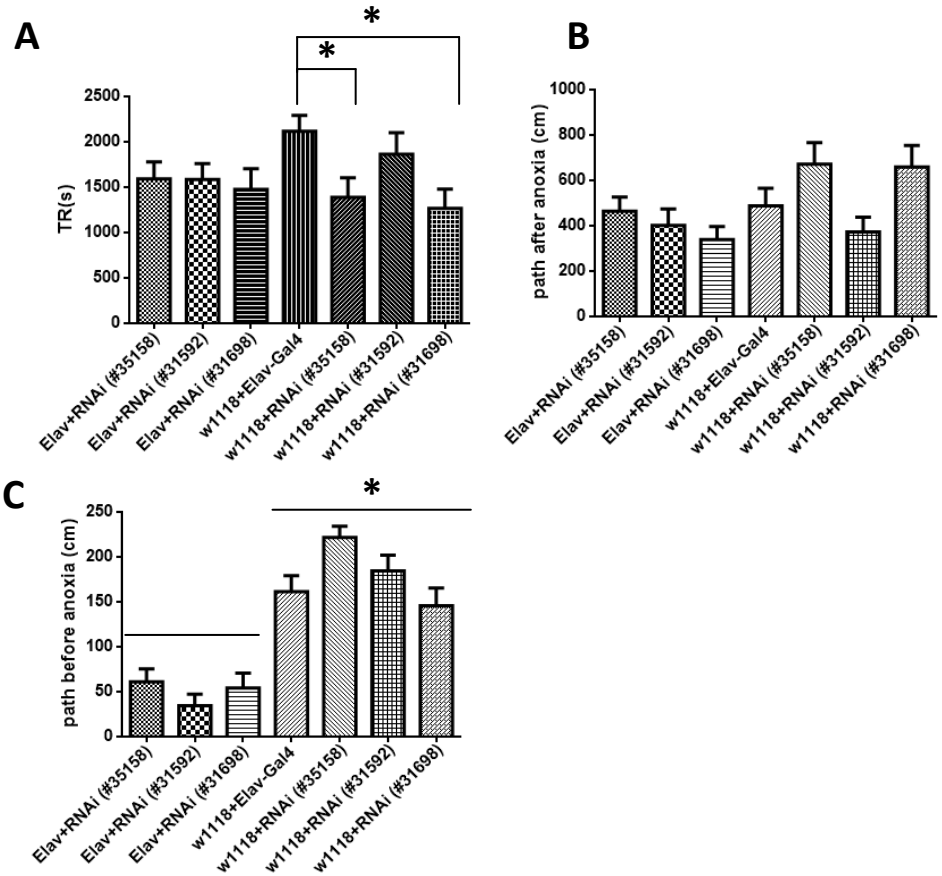

Figure 7. Results for recombinant and control flies. A comparison between strains for path length before and after anoxia is shown. Sample sizes (from left to right): 25, 17, 14, 35, 24, 23, 22. Error bars show standard error.

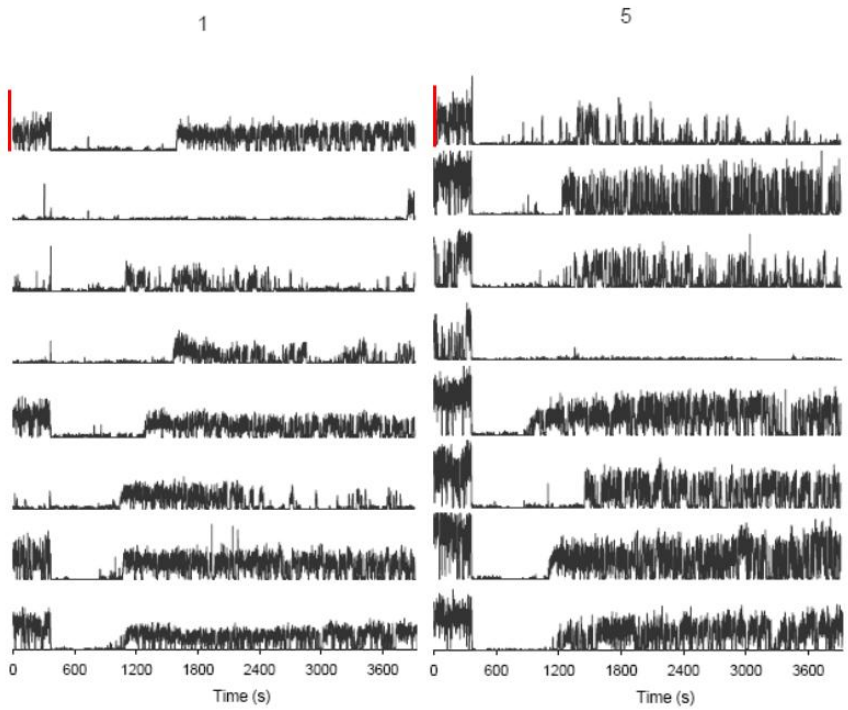

Figure 8: comparison between initial activity between strain 1 (RNAi 35158 x elav-Gal4) and 5 (RNAi 35158 x w1118). The red bar placed in the beginning of the graphs gives an idea of the difference in initial activity between the two strains. Each row represents the activity of one fly.

Discussion

PKG has been shown to play an important role in diverse physiological processes such as lipolysis, calcium homeostasis, smooth muscle contraction and platelet formation (Francis et al., 2010). It has also been shown to participate in the response to anoxic stress in *Drosophila melanogaster*, where higher PKG levels seem to provide a higher protection to anoxic stress, as the survival of rover flies after a long exposure to anoxia is considerably higher than that of sitter flies (Dawson-Scully et al., 2010; Wingrove and O'Farrel, 1999).

By analyzing the effects of a short exposure to anoxia of 30 seconds, this project was trying to complement these findings. The results collected for the foraging variants provided interesting information that added to the previous studies.

The comparison of the time to recovery between strains gave confusing results as for^{S} flies showed a longer time to recover even if for^{S2} flies, which have the same PKG levels but a rover genetic background, showed very similar TR to CS flies. However, this high TR in for^{S} flies could be due to the criteria used for the data analysis; flies that showed activity before anoxia but did not recover in the whole time of analysis were taken into account in the analyisis assuming the

maximum time to recover (3630 s). This was the case of 5 flies (15% of all the for^{S} flies), while that assumption was not made with any the flies from the other strains. This may have inflated the mean TR value for this strain.

However, the analysis of the 60 min path after anoxia and the last 10 min path gave interesting and consistent results. Flies with lower PKG (for^{S2}) showed significantly lower activity levels after anoxia than the rest of strains; comparing these results with a control assay performed without an anoxic treatment in which the flies kept a constant level of activity during the whole assay, we concluded that that decay in activity was a result of the anoxic stress. The reduced activity after anoxia was considered to be an indicator of poor recovery; thus, the lower activity in for^{S2} flies was associated to lower recovery in this strain. This agrees with previous studies about PKG effects on anoxia tolerance, in which sitter flies showed lower survival to prolonged anoxia than rovers. Those studies also analyzed the time to onset an anoxic coma under hypoxic conditions, and found that rover flies experienced it earlier than sitters (Dawson-Scully et al., 2010). This parameter could not be tested using our method; the onset of the coma happened within a few seconds of the oxygen removal in all the strains and no differences could be detected between them using only the behavioral analysis.

Therefore, PKG promotes the onset of an anoxic coma, which is believed to have a neuroprotective role, and thus, flies that enter it earlier (rover flies) recover better. Similar conclusions to this have been made regarding thermotolerance in *Drosophila melanogaster*, where lower PKG levels were associated to higher thermotolerance (longer time for failure of mouth hook movements and synaptic transmission) but a worse recovery (Dawson-Scully et al., 2007).

These results, together with previous studies examining the role of PKG on tolerance to abiotic stress (i.e. tolerance to anoxia and thermotolerance), support the hypothesis of an adaptive significance of developing different strategies to cope with abiotic stress. High PKG levels are related to higher activity in foraging behavior, as well as a more efficient metabolism of glucose (larger absorption and homeostasis), and greater ability to find food (higher sucrose responsiveness, ability to associate odors to presence of food) compared to low PKG levels. Therefore, when exposed to adverse environmental conditions (which, in nature, could be a decrease in oxygen or increase in temperature due to fermentation of the food) flies with high PKG levels are more likely to escape from them and thus a high resistance to the onset of an anoxic coma lacks adaptive significance; on the other hand, flies with lower PKG levels benefit from high resistance to the onset of an anoxic coma, which allows them to remain longer in the food patch. Their fitness after a coma, though, will be lower due to the initial reluctance to enter it, for SD is believed to have a neuroprotective role.

However, the results obtained with for^{R}, for^{S} and for^{S2} could not be verified in the experiments using the PKG mutants and RNA_i/Elav-Gal4 recombinants. These flies showed very low basal activity, even before exposure to anoxia, so no conclusions could be made specifically about their response to abiotic stress. All the PKG mutants showed lower activity before anoxia,

suggesting that the mutation introduced in the foraging gene affects the health of the flies independently from the exposure to anoxia. In RNAi recombinants, lower basal activity was observed in the RNAi x Elav-Gal4 crossings than in the RNAi x w1118 or Elav-Gal4 x w1118 controls, even if no differences were seen in time to recovery or level of activity after anoxia. This decreased fitness was most likely related to too low PKG levels (lower than those due to natural variation in the foraging gene); as PKG is an important protein that participates in many vital processes, a drastic decrease in its activity has very complex effects in physiology that make it difficult to make conclusions about its specific effects in anoxia recovery. Further experiments quantifying PKG mRNA in those strains using PCR could provide interesting information to correlate PKG mRNA levels with activity.

In conclusion, even if the results of the assays with natural foraging variants look promising, further testing should be done in the future to confirm these results and reinforce the conclusions made.

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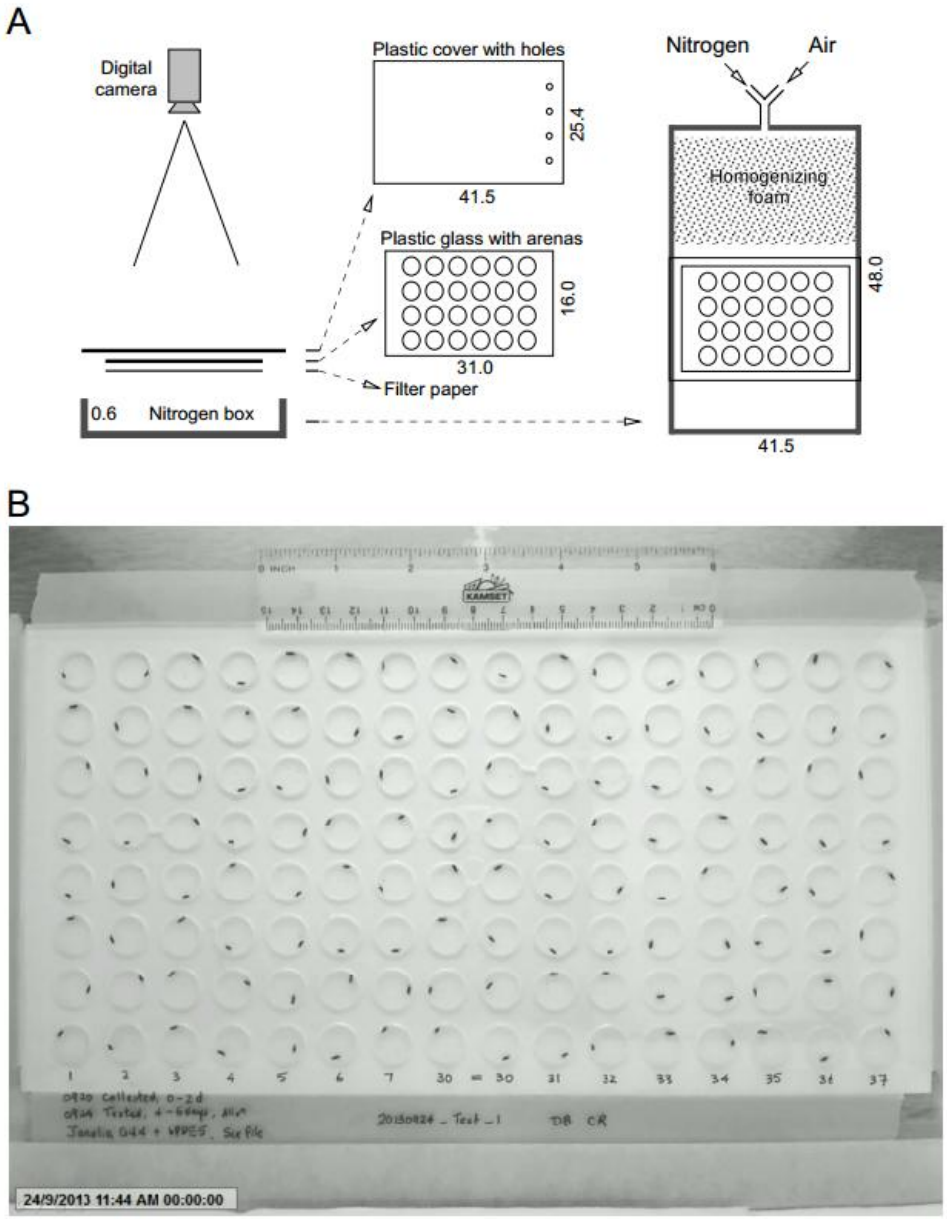
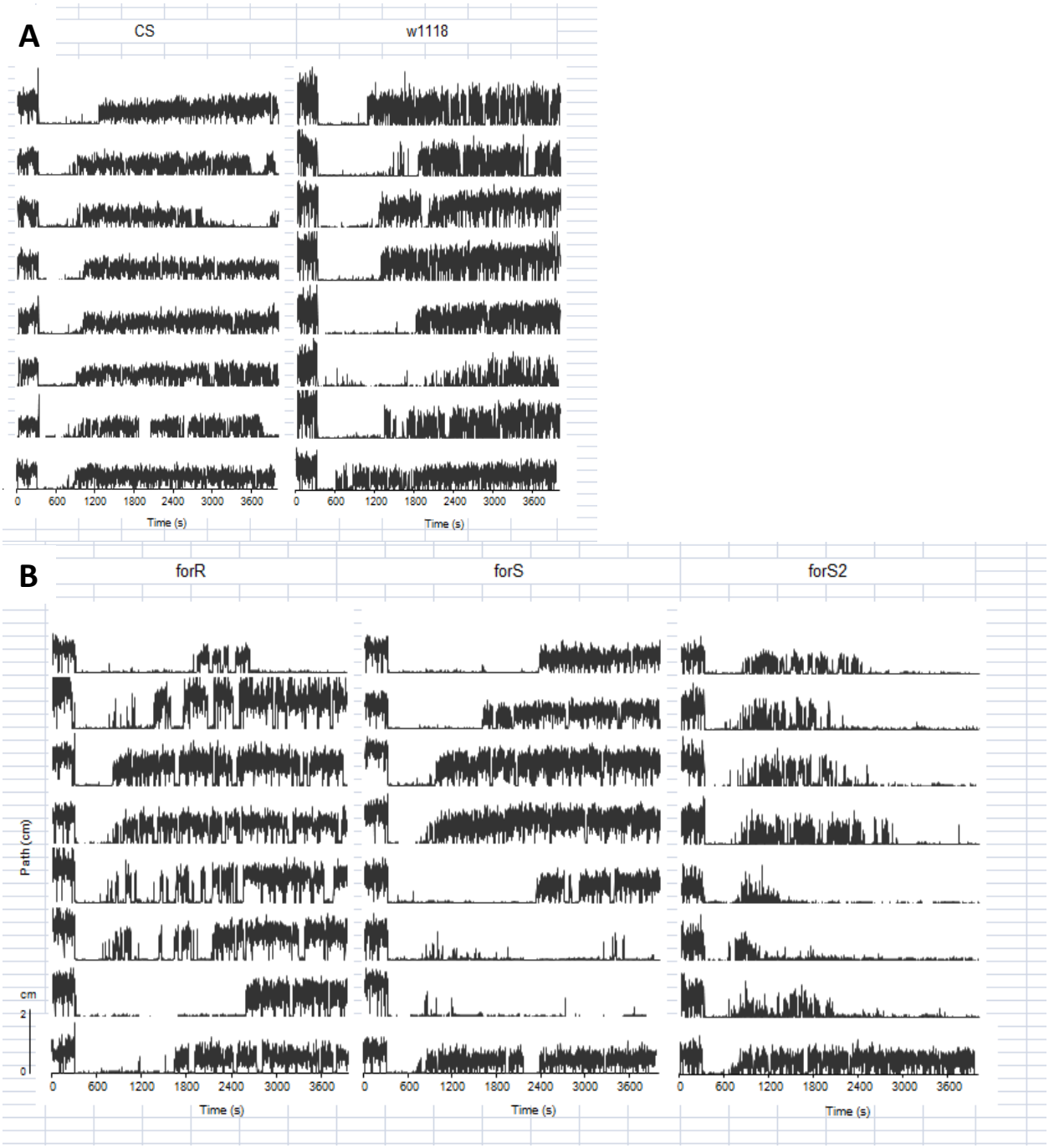
Supplemental figures



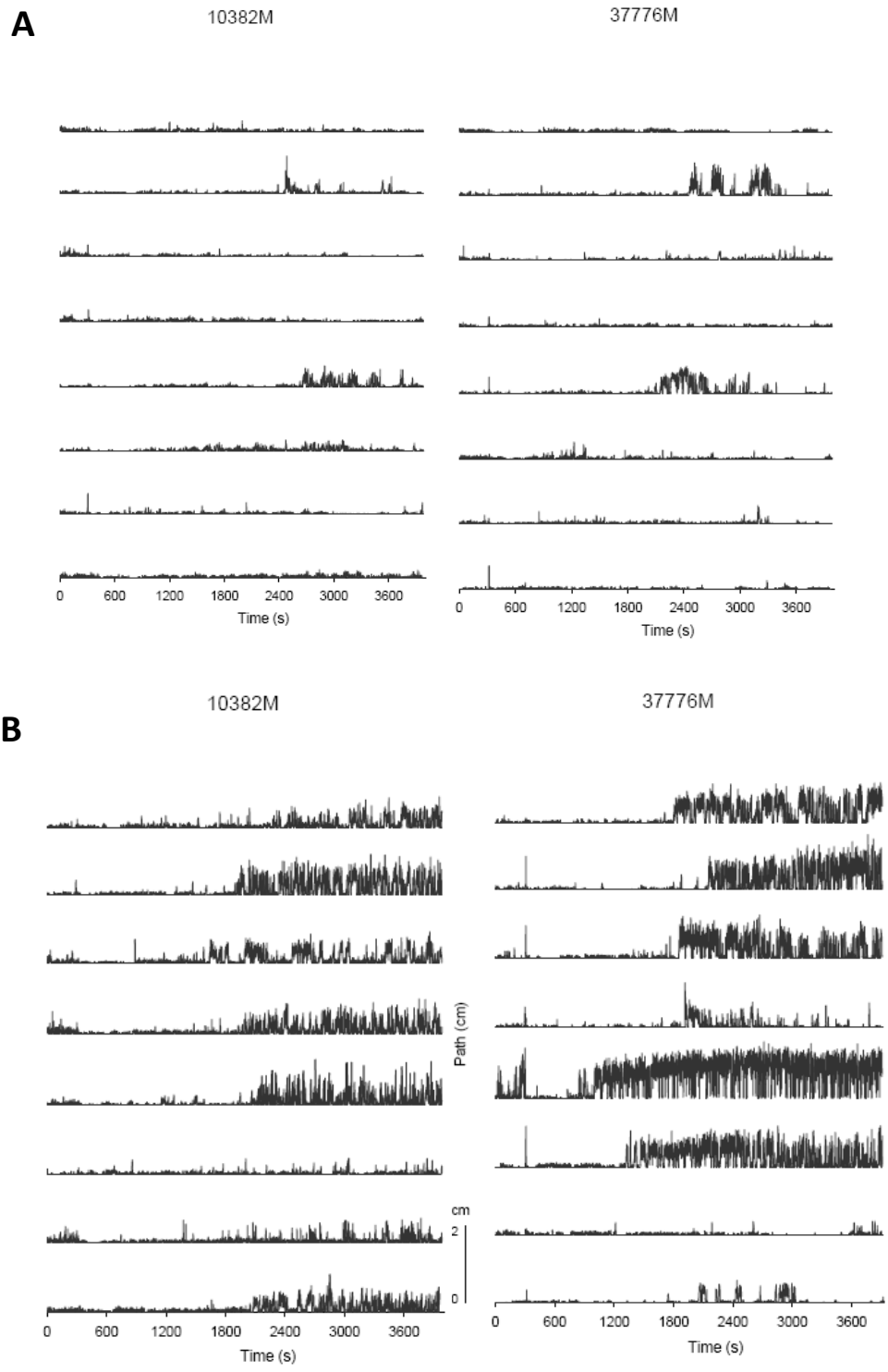
Supplemental figure 1. Experimental setup (Donated by C. Xiao). A: Representation of the setup, showing the entrances for N_2 and room air and the homogenizing foam that provides an equal supply of air to all the arenas, as well as the arrangement of the arenas for the flies, and

the camera. B: image of a fully loaded plaque of 8x16 arenas. Note that there is a single fly in each arena, and that all the flies in each column belong to the same strain.



Supplemental figure 2. Level of activity on the different strains over time. The graphs show an initial active period, an inactive period due to anoxic coma and recovered activity after exposure to anoxia for A: CS and w1118 (control) and B: foraging variants. Each of the rows represent the activity of one fly. Note big variance in the pattern of activity of for^{R}, for^{S} and

for^{S2} compared to the more reproducible data in the control strains. Also note the decrease in activity in most of the for^{S2} flies after recovery from the anoxic coma.



Supplemental figure 3. Comparison of the strains 10382 and 37776 between the second (A) and the fourth (B) assays.