Letter to the Editor:

Comment on: “Water- and nutrient-dependent effects of dietary restriction in *Drosophila* lifespan” by Ja et al, PNAS, doi: 10.1073

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Dietary restriction (DR) is a moderate reduction in food intake, without malnutrition, that extends healthy lifespan in many organisms, including *Drosophila* (1). Although fruitflies have many practical advantages for studying DR, various technical complexities can have large effects on experimental outcomes (1).

For DR, it is important that the basic food conditions are optimal. This ensures that increased longevity due to food restriction prolongs healthy lifespan rather than returning sick animals to normal health by limiting access to a nutritionally inappropriate diet. We have systematically optimised our conditions for *Drosophila* DR, eliminating several non-nutritional explanations including water imbalance, and recommend a Brewer's-yeast-based diet (2, 3). Ja et al. (4) report contradictory data. Here, we present additional data in support of our conclusions, and point out a flaw in the data concerning the Brewer's yeast diet presented by Ja et al.

First, we developed a system that effectively hydrates flies under salt stress (Table 1, experiment 1). Adding 8g.l^{-1} NaCl to our standard food (1.0 SYBrewer's (2)) shortened median lifespan by 24%. Adding to each vial a 200ul pipette tip filled with water (1% agar) restored normal lifespan. The rescue was not due to the tip itself, because an identical tip filled with dry cotton wool had no effect. Furthermore, addition of a tip containing wet cotton wool also restored normal lifespan. This was reproducible when the food was made from a different yeast (SYBaker's (2)) with salt added (Table 1, experiment 2). Thus, our technique is effective in delivering water to salt-stressed flies, and rescuing the associated lifespan shortening.

Next, we established that the lifespan change associated with DR was not rescued by water addition (Table 1, experiment 3; direct replication of our published data (2)).These data demonstrate that DR in *Drosophila* under our conditions is not due to rescue of hydration stress.

These results directly contradict data from an unreplicated experiment by Ja et al. using conditions ostensibly replicating ours, which reported that lifespan-extension by DR was eliminated by water addition, concluding that hydration stress explains DR (Fig 2G-I in ref (4)). There are two problems with this conclusion. First, Ja et al. used more sugar (100 g.l^{-1}) in their concentrated medium (CM) than did we, and this higher concentration causes a significant reduction in egg laying compared with the level we use (50 g.l^{-1}) (2), perhaps because of water stress[1]. Second, the data in Fig 2G-I, from Ja et al., demonstrate that water addition *shortened* DR lifespan to the level of CM (Figure 1). Thus the 'rescue' of the DR effect by water addition could equally be explained by water shortening the lifespan of DR flies, rather than increasing the lifespan of those on CM.

These data presented from our laboratory, both here and elsewhere (2, 3), robustly demonstrate that hydration stress does not explain DR under the conditions we use. Furthermore, we have discovered that DR in *Drosophila* is mediated by an amino acid imbalance in the food which is not modified by water addition (3).

[1] Furthermore, fly feeding behaviour can be dramatically reduced as sugar concentration increases in this range, which may explain why Ja et al see lowered feeding in their CM (Fig 1F)(4) and we do not.

1. Piper,MD, Partridge,L (2007) Dietary restriction in *Drosophila*: Delayed aging or experimental artefact? *PLoS. Genet.* 3, e57.

2. Bass,TM, Grandison,RC, Wong,R, Martinez,P, Partridge,L *et al.* (2007) Optimization of dietary restriction protocols in *Drosophila J. Gerontol. A Biol. Sci. Med. Sci.* 6, 1071-1081.

3. Grandison,RC, Piper,MDW, Partridge,L (2009) Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila *Nature* doi:10.1038/nature08619.

4. Ja,WW, Carvalho,GB, Zid,BM, Mak,EM, Brummel,T *et al.* (2009) Water- and nutrient-dependent effects of dietary restriction on Drosophila lifespan *Proc. Natl. Acad. Sci. U. S. A.* 106, 18633-18637.

Figure 1. Piper et al

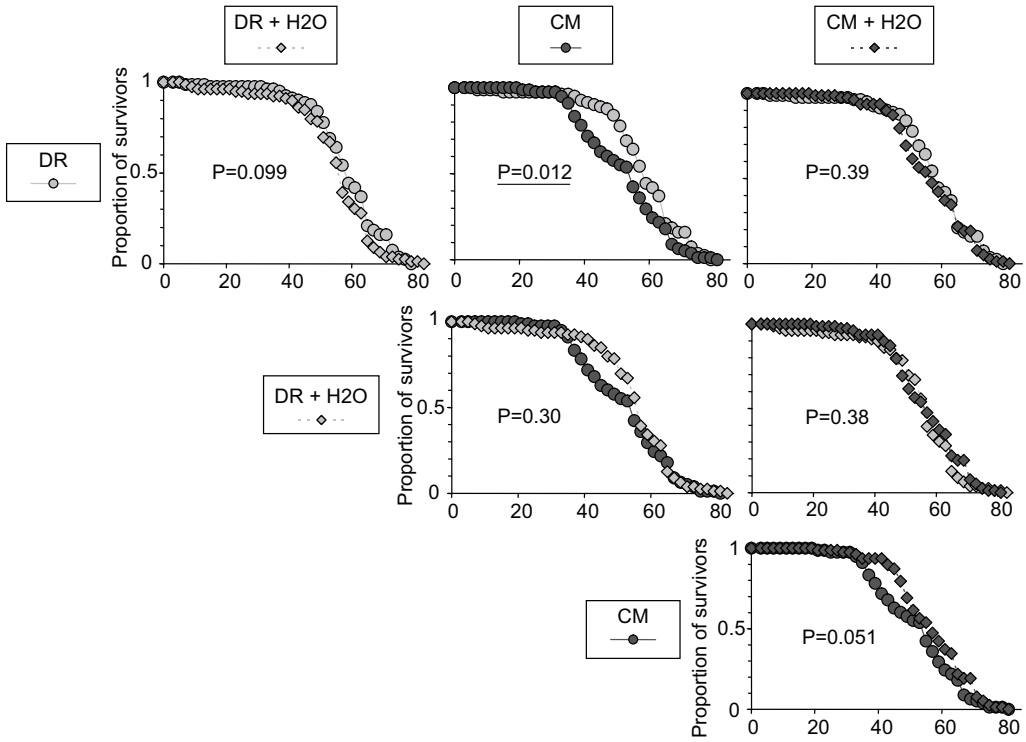

Figure 1. Lifespan differences due to DR and water addition.
Ja et al (4) reported significant extension of lifespan when the concentration of nutrients in a Brewer's yeast diet were diluted (DR v CM) and that this difference was eliminated by water addition (DR+H2O v CM+H2O). Further comparisons show that this could be caused by water extending the lifespan of CM flies (DR v CM+H2O) or water shortening the lifespan of DR flies (DR+H2O v CM). Data supplied by W.W. Ja. P values generated using the Log rank test.

Table 1. Providing a water source to flies rescues the life shortening effect caused by salt (NaCl) addition to food, but not lifespan alterations due to dietary restriction.

Experiment 1: Brewer's yeast[1][2]						No salt	+ salt (137mM)				
	sugar (g/l)					50	50	50	50	50	
		yeast (g/l)				100	100	100	100	100	
			Salt (NaCl) (g/l)			0	8	8	8	8	
				Water?[3]		None	None	Dry cotton	Wet cotton	1% agar tip	
					median LS	55	42	42	52	53	
No salt	50	100	0	None	55		-24%[4] P<0.001	-24% P<0.001	-5% P<0.001	N.C. P=0.16	
+ salt (137mM)	50	100	8	None	42			N.C. P=0.08	+24% P=0.004	+26% P<0.001	
	50	100	8	Dry cotton	42				+24% P<0.001	+26% P<0.001	
	50	100	8	Wet cotton	52					N.C. P=0.06	
	50	100	8	1% agar tip	53						

Experiment 2: Baker's yeast[5]						+ salt (137mM)					
	Sugar (g/l)					100	100	100			
		Yeast (g/l)				100	100	100			
			Salt (g/l)			8	8	8			
				Water?		None	Dry cotton	1% agar tip			
					Median LS	42	42	53			
+ salt (137mM)	100	100	8	None	42		N.C. P=0.08	+26% P<0.001			
	100	100	8	Dry cotton	42			+26% P<0.001			
	100	100	8	1% agar tip	53						

Experiment 3: Brewer's yeast		Dietary restriction			Fully-fed		
	Sugar (g/l)	50	50	50	50	50	50

[1] all experiments were performed on 100 female flies per condition (10 vials of 10 flies each). Flies were reared and prepared for lifespan experiments as previously described (Bass et al., 2007)
[2] MPBiomedicals, USA; recipe as described for SYBrewer's in (Bass et al., 2007)
[3] to deliver water to flies, a single 200uL filter-barrier pipette tip was stabbed into the food of each vial. This was either filled with 1% agar made in water, or dry cotton wool (Dry cotton) or cotton wool soaked in water (Wet cotton). Dry cotton wool was added to avoid the problem of flies climbing into the pipette tip and not being able to get out.
[4] percentage change is based on a comparison of median lifespans; median lifespan of conditions in upper panels divided by those in the left panels; data in red indicate statistically significant differences (P<0.05, log rank test)
[5] BTP Drewitt, UK

		Yeast (g/l)				100	100	100	200	200	200
			Salt (g/l)			0	0	0	0	0	0
				Water?		None	Dry cotton	1% agar tip	None	Dry cotton	1% agar tip
					Median LS	62	62	60	41	46	41
Dietary restriction	50	100	0	None	62		N.C. P=1	N.C. P=0.8	-34% P<0.001	-34% P<0.001	-26% P<0.001
	50	100	0	Dry cotton	62			N.C. P=0.7	-32% P<0.001	-32% P<0.001	-23% P<0.001
	50	100	0	1% agar tip	60				-34% P<0.001	-34% P<0.001	-26% P<0.001
Fully-fed	50	200	0	None	41					N.C. P=0.7	N.C. P=0.1
	50	200	0	Dry cotton	46						-10% P=0.04
	50	200	0	1% agar tip	41						