Review of: "Verification of Hypsibius exemplaris Gąsiorek et al., 2018 (Eutardigrada; Hypsibiidae) application in anhydrobiosis research"

Kazuharu Arakawa[1]

1 Keio University

Potential competing interests: The author(s) declared that no potential competing interests exist.

Poprawa et al. tests three drying protocols to test the survival of a tardigrade *Hypsibius exemplaris* from anhydrobiosis, and concludes that its survival rate is only around 50% and therefore the species “appears not to be a good model species for anhydrobiosis research”. *Hypsibius exemplaris* (formerly known as *Hypsibius dujardini* Z151, a strain sold by Sciento) is one of the most widely used species in tardigrade research, but its desiccation tolerance is not as strong as other strong anhydrobiotes such as *Ramazzottius varieornatus*, *Milnesium inceptum*, or *Echiniscus testudo*. While these strong anhydrobiotes can immediately (in the order of 15~30 min) enter anhydrobiosis, *H. exemplaris* requires a preconditioning period of ~24h before entering anhydrobiosis, during which *H. exemplaris* expresses hundreds of proteins required for successful anhydrobiosis. Therefore, while not a strong anhydrobiote, *H. exemplaris* is nevertheless a useful model species to be compared with other closely related tardigrades with strong desiccation tolerance, such as *Ramazzottius varieornatus* (Yoshida et al. 2017 PLoS Biol) leading to the identification of hundreds of genes presumably participating in the molecular machinery of anhydrobiosis.

The main claim of this work, that the survival rate of *H. exemplaris* anhydrobiosis is only 50% is extremely challenging. The first and most comprehensive work regarding anhydrobiosis in H. exemplaris was Kondo et al. (2015) PLoS One, in which the necessity of ~24h “preconditioning” at relative humidity > 85% before entering anhydrobiosis is clearly shown, and that de novo gene expression taking place during this period is essential. Kondo et al. showed 90~100% survival rate consistently in numerous experiments, clearly showing the anhydrobiotic capability of this species.

A number of works follow Kondo et al. (2015) PLoS One from different labs, well replicating the anhydrobiosis of *H. exemplaris*.

- Arakawa et al. (2016) Sci. Data, >90% recovery rate
- Boothby et al. (2017) Mol. Cell, >80% survival
- Yoshida et al. (2017) PLoS Biol, >90% recovery rate
- Kondo et al. (2019) Genes Cells, >90% survival
- Kondo et al. (2020) FEBS Open Bio, >90% survival

- Arakawa and Numata (2021) Mol. Cell, >90% recovery rate

Preconditioning of *H. exemplaris* anhydrobiosis is simple, but requires certain proficiency in its handling. Firstly, one needs to understand that this process is a “preconditioning” step, and not a “dehydration” procedure. As noted above, extensive de novo expression of hundreds of proteins take place during the preconditioning step, many of which are induced x10~1000 fold (Kondo et al. 2015 PLoS One, Yoshida et al. 2017 PLoS Biol), so the body water content MUST stay unchanged in order for the biochemistry of transcription and translation machineries to remain unaffected. As the pineering work by Wright (1989) J. Exp. Biol. clearly demonstrates in its Table 1, survival of *H. dujardini* to dehydrating environment for just one hour sharply drops at relative humidity below 85%. Therefore, the preconditioning step MUST NOT introduce dehydration. On the other hand, chemical preconditioning with D942 by soaking *H. exemplaris* in this solution for 24h allows the tardigrade to enter anhydrobiosis by dehydating it in low humidities (Kondo et al. 2020 FEBS Open Bio). Therefore, the preconditioning requires hydration - at least a thin water layer must always surround the tardigrade - to allow the tardigrade to undergo extensive gene expression, and only after the preconditioning, and with necessary proteins highly expressed, can the tardigrades be dehydrated. This is why, the successful preconditioning protocols in the above-listed works employ high relative humidity chambers (at least > 90%, desirably higher such as >95% or >97%) to suppress water evaporation, and keep the tardigrades on moist substrate (wet filter paper, or agarose medium) so that a thin water layer is sustained throughout the 24h preconditioning period.

Three dehydration protocols used in Proprawa et al. are:

A. preconditioning on agar plates at 92% RH and drying at 40%RH

B. drying on filter paper at 40-50% RH

C. drying on sands or pond sediments at 40-50% RH

and only B showed round 50% recovery. I presume that the water retention on agarose with minimal amount of initial water at 92% RH was not sufficient to keep the water layer around the tardigrade for the entire course of preconditioning of 16h (note that which is also shorter than other previous works). In B, 400µl of water is initially applied, which chould have been retained better on filter paper than agarose, allowing precondition in some of the individuals, but 40-50% RH was too dry and dehydrated other individuals. Therefore, I suspect that protocols of Poprawa et al. did not properly precondition *H. exemplaris*, and that the low anhydrobiotic survival rate is not because the species is not a good model for anhydrobiotic research.

Subsequent ultrastructure analysis show very similar percentage of intact storage cells (~50%) to the recovery rate (~50%), and thus little cellular damage would be observed if these tardigrades are properly preconditioned. On the other hand, deformed mitochondria is observed during recovery from anhydrobioisis in a strong anhydrobiote *Ramazzottius varieornatus* (with nearly 100% anhydrobiotic survival, Yoshida et al. (2020) bioRxiv) as in H. exemplaris (Richaud et al. 2020 Sci Rep), which is an interesting indicator of possible mitochondrial oxidative stress.

On minor note, Poprawa et al. (submitted Nov. 28 2021) states “At the present, the genomes of only two tardigrade species are available i.e. *Hypsibius exemplaris* Gąsiorek, Stec, Morek & Michalczyk, 2018 [27] (in earlier works misidentified as *Hys. dujardini* (Doyère, 1840) [28] and *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 [29–32], both representing the eutardigrade lineage”, but there are also the genome of *Paramacrobiotus sp.* (Hara et al. 2021 Open Biol, published Jul. 14 2021) and that of *Echiniscus testudo* (Murai et al. 2021 BMC Genomics, published Nov. 11 2021).