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Joanne M. Tang
Western University, jtang283@uwo.ca

Yanira Jiménez Padilla
Western University, yjimenez@uwo.ca

Marc-André Lachance
Western University, lachance@uwo.ca

Brent J. Sinclair
Western University, bsincla7@uwo.ca

Gut yeasts do not improve desiccation survival in *Drosophila melanogaster*

Joanne M. Tang[1], Yanira Jiménez-Padilla, Marc-André Lachance, and Brent J. Sinclair*

Department of Biology, University of Western Ontario, London, ON, Canada N6G 1L3

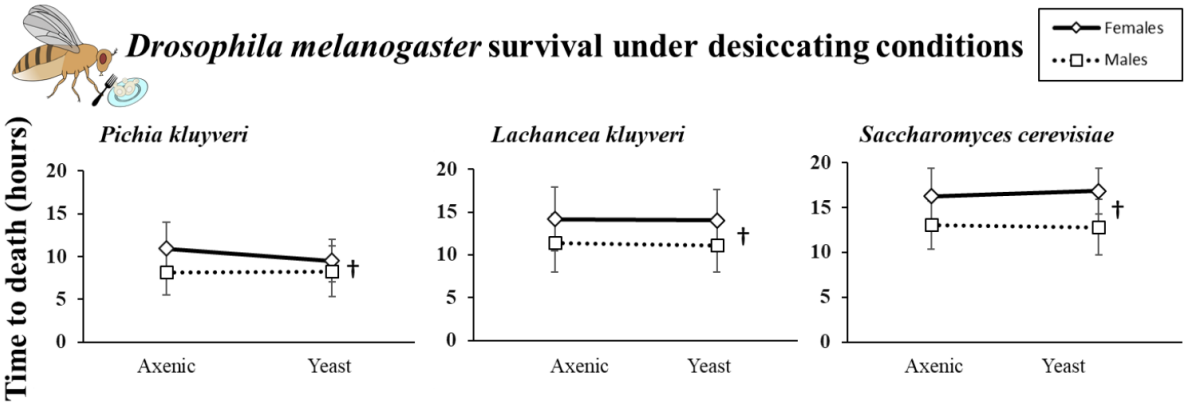
[1]Present Address: Department of Medical Biophysics, University of Western Ontario, London, ON, Canada.

*Corresponding Author: email bsincla7@uwo.ca, tel: +1-519-661-2111 x83138

Research Highlights:

- Gut yeasts do not improve adult *Drosophila melanogaster* desiccation survival
- Rearing flies with non-coevolved yeasts reduces adult desiccation survival
- *Saccharomyces cerevisiae* may not be a beneficial contributor to the gut microbiota

Graphical Abstract:



Abstract

A healthy gut microbiota generally improves the performance of its insect host. Although the effects can be specific to the species composition of the microbial community, the role of gut microbiota in determining water balance has not been well-explored. We used axenic and gnotobiotic (reared with a known microbiota) *Drosophila melanogaster* to test three hypotheses about the effects of gut yeasts on the water balance of adult flies: 1) that gut yeasts would improve desiccation survival in adult flies; 2) that larval yeasts would improve adult desiccation survival; 3) that the effects would be species-specific, such that yeasts closely associated with *D. melanogaster* in nature are more likely to be beneficial than those rarely found in association with *D. melanogaster*. We used *Saccharomyces cerevisiae* (often used in *Drosophila* cultures, but rarely associated with *D. melanogaster* in nature), *Lachancea kluyveri* (associated with some species of *Drosophila*, but not *D. melanogaster*), and *Pichia kluyveri* (associated with *D. melanogaster* in nature). Adult inoculation with yeasts had no effect on survival of desiccating conditions. Inoculation with *P. kluyveri* as larvae, did not change desiccation survival in adults; however, rearing with *L. kluyveri* or *S. cerevisiae* reduced adult desiccation survival. We conclude that adult inoculation with gut yeasts has no impact on desiccation survival, but that rearing with yeasts can have either no or detrimental effect. The effects appear to be species-specific: *P. kluyveri* did not have a negative impact on desiccation tolerance, suggesting some level of co-adaptation with *D. melanogaster*. We note that *S. cerevisiae* may not be an appropriate species for studying the effects of gut yeasts on *D. melanogaster*.

Key Words: Desiccation; gut microbiota; phenotypic plasticity; *Pichia kluyveri; Lachancea kluyveri; Saccharomyces cerevisiae*

Abbreviations:

FM: Fresh mass

MAD: Mass at death

DM: Dry mass

IWC: Initial water content

WCD: Water content at death

WLR: Water loss rate

1. Introduction

Animals have an abundant and diverse microbiota that can have profound impacts on their physiology, reproduction, and behaviour (McFall-Ngai et al., 2013). Although most epithelia have a distinct microbiota, and some microbial symbionts are intracellular, a majority of microbes colonise the gut (Douglas, 2018b). Insects are tractable models for studying the effects of this gut microbiota on performance (Douglas, 2015), not least because of the ease with which aseptic rearing and inoculation can yield axenic (no microbiota) and gnotobiotic (with a known microbiota) individuals. The gut microbiota has profound effects on digestion (Peterson and Scharf, 2016), toxin breakdown (Welte et al., 2016), immunity (Dillon and Charnley, 1986), nutrition (Scully et al., 2014) and behaviour (Wada-Katsumata et al., 2015), and can modify development time and cold tolerance (Jiménez Padilla, 2016). These effects can be context-dependent. For example, microbe inoculation during development increases *Drosophila* lifespan, but inoculation as an adult reduces lifespan (Brummel et al., 2004). Moreover, some gut microbes have a greater impact than others (Douglas, 2018b; Newell et al., 2014). In general, however, those individuals with a gut microbiota tend to outperform axenic individuals that lack gut microbes (Douglas, 2015; but see Henry and Colinet, 2018; Judd et al., 2018).

The gut microbiota of insects includes bacteria, archaea, viruses, and yeasts (Douglas, 2015). While a majority of studies have focused on bacteria (Engel and Moran, 2013), there is a rich yeast community associated with insects, including with *Drosophila* spp. (e.g. Anagnostou et al., 2010; Lachance et al., 1995; Starmer and Fogleman, 1986). More than 56 species of yeasts have been identified from the guts of wild-caught *Drosophila* spp. (Lachance et al., 1995); however *Saccharomyces cerevisiae,* the standard yeast used in *Drosophila* spp. rearing (Ashburner et al.,

2005; Markow and O'Grady, 2005), is not one of the species regularly identified in the guts of wild-caught flies (Chandler et al., 2012) and may not be a *D. melanogaster* symbiont (Hoang et al., 2015). Jiménez Padilla (2016) showed that yeasts primarily colonise the guts of female *D. melanogaster*, that *S. cerevisiae* persisted in the gut for less time than *Lachancea kluyveri* [a yeast collected from *D. robusta, D. pinicola,* and *D. algonquin* in the wild (Lachance et al., 1995; Phaff et al., 1956)], and that *L. kluyveri* had a larger effect on *D. melanogaster* chill coma recovery time than *S. cerevisiae*. Thus, just as with gut bacteria (Newell et al., 2014), the phenotypic effects of gut yeasts on *Drosophila* may be species-specific, and may not stem only from the additional nutrients acquired from digesting the yeast, as has previously been suggested (Anagnostou et al., 2010; Shin et al., 2011).

The ability to maintain water balance under desiccating conditions has been key to the success of terrestrial insects (Harrison et al., 2012). Insects that survive desiccating conditions for longer generally use some combination of increased initial water content, reduced water loss rate, and tolerating the loss of more water (Gibbs, 2002; Gibbs et al., 1997; Gibbs and Matzkin, 2001). *Drosophila* spp. that best survive desiccation, or populations of *D. melanogaster* selected to survive for longer, use the first two of these strategies. Water loss rate across the cuticle can be modified by changing the cuticular hydrocarbons to yield plasticity even within a population (Bazinet et al., 2010; Hoffmann, 1990; Stinziano et al., 2015). Dung beetles reared under desiccating conditions have better survival, faster growth rates, and larger final body size when they have a full, rather than reduced, microbiota (Schwab et al., 2016), although the mechanisms underlying this difference were not determined. Thus, the role – if any – of the gut microbiota (and especially yeasts) on insect water balance remains largely unexplored.

Here we examined the effects of gut yeasts on desiccation survival of adult *D. melanogaster* to better understand the role of gut yeasts in insect water balance. First, we hypothesised that gut yeasts improve desiccation survival. We compared the survival time of axenic and gnotobiotic flies under desiccating conditions, and measured water content, desiccation tolerance, and water loss rates to examine the mechanisms underlying any differences. Second, we hypothesised that development with gut yeasts improves desiccation survival in adults. We reared axenic and gnotobiotic larvae, and then compared their survival as adults under desiccating conditions. Finally, we hypothesised that the effects of the gut community on performance under desiccating conditions are species-specific. We compared gnotobiotic flies inoculated with *Pichia kluyveri* [which is regularly found in *D. melanogaster* guts in nature (Lachance et al., 1995)], *Lachancea kluyveri* [which has a strong phenotypic effect on *D. melanogaster* development time and cold tolerance (Jiménez Padilla, 2016), but is not normally found in *D. melanogaster* in nature], and *Saccharomyces cerevisiae*, which is seldom found in *D. melanogaster* in nature, but is an ubiquitous additive in laboratory *Drosophila* cultures (Ashburner et al., 2005; Markow and O'Grady, 2005).

2. Methods

2.1 Study Organisms

We used an outbred, wild-type *Drosophila melanogaster* population collected in London, Ontario, Canada (43º00´N, 81º15´W) in 2007 (Marshall and Sinclair, 2010). We reared the flies in 35 mL vials containing 20 mL of Tucson fly food [1 L dH_2O, 45 g sugar, 30 g cornmeal, 18 g dry active *S. cerevisiae* (Fleischmann's Yeast, Farinex, QC, Canada), 12 g agar; adapted from Markow and O'Grady (2005)] under standard long-day conditions (21.5 ± 1 °C, 60 ± 5 % RH, 13 h:11 h L:D) at standardized densities (50 eggs/vial). We transferred three- to five-day old adult flies into small oviposition cages (⌀= 3.5 cm, h = 5.8 cm) capped with a Petri dish of apple juice-agar (100 mL fruit juice, 100 mL dH_2O, 4 g agar) topped with yeast paste (inactive yeast and distilled water) for three days. For egg collection, we replaced the yeast-topped apple-agar plates with apple-agar plates on which the flies laid eggs for 12 h.

To generate axenic flies, we transferred fifty eggs onto autoclaved sterile 20 μm pore nylon filters (50 eggs/filter; ⌀ = 24 mm, NY2002500, EMD Millipore), and surface-sterilized them by submerging them in 70 % ethanol for 5 min and rinsing them with autoclaved phosphate buffered saline (PBS) (Sigma-Aldrich, P4417, prepared as per manufacturer's instructions). Each filter was then inverted onto a sterile yeast-sucrose agar plate (1.5 g agar, 1.5 g active yeast, 4.3 g sucrose, 100 mL dH_2O) to separate the eggs from the filter. We then cut the agar into pieces containing the eggs (still 50/vial) and transferred the pieces to autoclaved 35 mL vials each containing 10 mL of Tucson fly food (the active yeasts in the recipe are killed when the food is autoclaved). Vials were incubated under standard conditions until adult emergence. We confirmed that the adult flies in each vial were axenic prior to use in experiments. We narcotized

flies with CO_2, and collected two flies of each sex and a small sample of food surface from each vial. We homogenized these samples in 100 μL sterile PBS, plated them on Yeast-Malt agar (YM agar) (1 % glucose, 0.5 % peptone, 0.3 % malt extract, 0.3 % yeast extract, 2 % agar), and incubated them at 25 ºC for 48 h. All flies reared in vials showing any microbial growth after 48 h were discarded from the experiment. In practice, we had 85-90 % success in maintaining sterile conditions, even though we had several instances of transferring flies. The remaining axenic flies were transferred into new 35 mL vials of sterile Tucson fly food for experiments.

To make adult-inoculated gnotobiotic flies, we took axenic adults, and allowed them to feed on live yeasts for 48 h; controls were provided with PBS or heat-killed yeasts at the same concentration. Yeasts were *Pichia kluyveri* (strain UWOPS 91-603.2), *Lachancea kluyveri* (strain NRRL.Y-12651), and *Saccharomyces cerevisiae* (strain UWOPS 92-222.2) obtained from the yeast collection of the Department of Biology, University of Western Ontario. We pipetted 10 μL of a suspension of 1.3×10^8 cells/mL in PBS (or 10 μL of sterile PBS, for controls) prior to adding axenic flies. Flies were allowed to feed on the yeast for 48 h at 21.5 ℃ prior to use in experiments. To confirm that the gnotobiotic flies were colonised with yeasts, we removed two male and two female flies from each yeast-inoculated vial, surface sterilised them in 70 % ethanol, rinsed them twice in sterile PBS, and then homogenized them in 100 μL of sterile PBS. We plated the homogenate on YM agar, incubated for 48-72 h at 25 °C, and checked for yeast growth (vials without yeast growth, or with noticeable bacteria growth, were removed from the experiment). The remaining flies from each vial were used in desiccation experiments. We reared flies under gnotobiotic conditions by adding 10 μL of yeast suspension into vials containing 10 mL of sterile Tucson food before transferring fifty surface-sterilised eggs to each

vial and incubating them under standard rearing conditions. Checks for contamination were conducted as described above, and contaminated vials discarded from the experiments.

As a control for the nutritional contribution of yeasts in the gnotobiotic treatment, we reared flies with heat-killed yeasts. We heat-killed yeast suspensions (1.3×10^8 cells/mL in PBS) by heating them at 60 °C for 30 min. We confirmed that the yeasts were dead by plating 10 μL of the suspension on YM agar plates before transferring fifty surface-sterilised eggs to vials containing 10 mL sterile Tucson food inoculated with 10 μL of heat-killed yeast suspension and incubating as above. As above, vials showing bacterial or yeast growth were discarded from the experiments.

2.2 Desiccation survival and water balance parameters

We measured survival under desiccating conditions as described by Gefen et al. (2006) and Bazinet et al. (2010). Briefly, we transferred flies individually into empty non-sterile 35 mL vials without anaesthesia (one fly per vial, n>28 flies per treatment, 28-33 vials/treatment, based on a power analysis from pilot experiments). We assumed that the duration of the desiccation experiment was too short for the flies to form a functioning gut microbiota from an empty vial, and that the mass lost was primarily water. We confined the fly to the bottom half of the vial using a foam stopper and added approximately 2 g of silica gel before sealing the vial with parafilm. We recorded survival (ability to right themselves) at hourly intervals until all flies were dead. To control for starvation, we separated 28-33 flies from different vials individually into vials containing water-agar medium and observed no mortality over the duration of the experiment, which suggests that handling and starvation did not cause mortality in either the

axenic or gnotobiotic. To control for variation between experimental days, experiments conducted on the same date were denoted with the same cohort number and we included cohort as a term in models.

We determined initial water content, water content at death, and water loss rate gravimetrically. Briefly, we weighed each fly (MX5 microbalance, Mettler Toledo, Columbus, OH, USA) before the start of the experiment to obtain fresh mass (FM), after the desiccation experiment to obtain mass at death (MAD), and after drying at 60 °C for 48 h to obtain dry mass (DM). Initial water content (IWC) was calculated as the difference between FM and DM; water content at death (WCD) as the difference between MAD and DM, and water loss rate (WLR) as the difference between IWC and WCD divided by time to death (yielding an average rate in mg H_2O/hour).

We compared survival time under desiccating conditions, initial water content, water content at death, and water loss rate between axenic and gnotobiotic flies separately for each yeast and treatment (reared or adult-inoculated) using two-way analyses of covariance (ANCOVA) in R (version 3.2.2), with dry mass, sex, and cohort (the date of the experiment) as covariates. We chose to use this linear model approach to compare survival times (cf. Gibbs et al., 1997) instead of a survival analysis (cf. Jakobs et al., 2015) as it allowed us to include cohort effects in our models. However, we provide survival curves and log-rank analyses, which are consistent with these results, in supplementary material). We included only individuals for which all measurements were available and discarded any for which measurement errors resulted in impossible values (e.g. negative water content). We corrected all p-values using a study-wide

Benjamini-Hochberg FDR correction (Benjamini and Hochberg, 1995) using the p.adjust function in R.

3. Results

Axenic flies were consistently sterile, and we were able to culture the expected yeasts from adult-inoculated gnotobiotic flies as well as gnotobiotic-reared flies (example culture plates in Figures S1 and S2). Female flies and larger flies survived desiccation significantly longer than males or smaller flies in all groups and treatments.

3.1 Gut yeasts do not improve desiccation survival in adult-inoculated flies

Neither female nor male *D. melanogaster* inoculated as adults with any of the species of yeasts survived desiccation stress significantly longer than their axenic counterparts (Figure 1; Table 1; Figs S3-S5), although a survival analysis (that considers the entire curve) showed that one cohort of *P. kluyveri*-inoculated female flies had worse survival than their axenic counterparts. There was no sex × treatment interaction, but there was a significant cohort effect, leading us to retain this term in our model.

Despite this overall lack of effects of yeasts on desiccation survival, gut yeasts did change some water balance parameters. Inoculation with any of the yeasts did not change initial water content (Figure 2A, D, G; Table 1). Water content at death was not significantly affected by any of the three yeast treatments (Figure 2B, E, H; Table 1). Inoculation with any of the yeasts had no significant impact on water loss rates (Figure 2C, F, I; Table 1).

3.2 Gnotobiotic rearing with yeast does not improve desiccation survival

Female and male adults reared with *P. kluyveri* did not have significantly different survival time under desiccating conditions than their axenic counterparts, but rearing with either *Lachancea kluyveri* or *S. cerevisiae* significantly decreased survival time under desiccating conditions compared to axenic flies in both females and males (Figure 3; Table 2; Figure S7). Flies reared with yeasts of all three species had significantly higher initial water contents than their axenic counterparts, even after accounting for the effects of body size and sex (Figure 4A, D, G; Table 2). However, the gnotobiotic flies reared with *L. kluyveri* and *S. cerevisiae* (but not *P. kluyveri*) had higher water loss rates than their axenic counterparts (Figure 4C, F, I; Table 2). In addition, both males and females of flies gnotobiotically-reared with *P. kluyveri* and *L. kluyveri* had higher water contents at death than their axenic counterparts, but there was no effect of yeast rearing on the water content at death of gnotobiotic flies reared with *S. cerevisiae* (Figure 4B, E, H; Table 2).

Rearing with heat-killed yeasts did not change the differences between male and female flies, but rearing with dead *S. cerevisiae* did slightly increase desiccation survival time in females (Figure 3; Table 3), although this was not significant in a survival analysis (Figure S8). Rearing with heat-killed yeasts increased water content at death in flies inoculated with both *P. kluyveri* and *S. cerevisiae*, decreased rate of water loss in *S. cerevisiae*, and slightly increased initial water content in flies reared with dead *S. cerevisiae*. However, rearing with heat-killed *L. kluyveri* had no impact on water balance parameters (Figure 5; Table 3).

4. Discussion

Investigations of the impact of gut microbiota on insect phenotypes have generally focused on bacteria (Douglas, 2018a), and in these studies, insects with a gut microbiota usually outperform their axenic counterparts (Coon et al., 2014; Wong et al., 2014). Here we investigate the role of gut yeasts on the water balance of *Drosophila melanogaster*. We find that inoculation with yeasts does not improve desiccation survival of adult flies, and that flies reared with live yeasts have reduced desiccation survival relative to their axenic counterparts. This impact is species-specific, and it appears that yeast species not usually associated with *D. melanogaster* in nature – including *S. cerevisiae,* which is widely used in *Drosophila* culture – are more likely to have a deleterious impact.

Desiccation survival by adult *D. melanogaster* is plastic at several timescales, ranging from brief 'hardening' responses (Bazinet et al., 2010; Hoffmann, 1990) to longer term responses to selection (Gibbs et al., 1997). Although live yeasts will colonise the guts of adult flies (Jiménez Padilla, 2016), we found no impact of inoculation with live yeasts on survival time under desiccating conditions. Inoculation with live *S. cerevisiae* did increase initial water content, but this was offset by a slightly increased water loss rate, yielding a survival time indistinguishable from axenic flies, or those inoculated with *L. kluyveri* or *P. kluyveri*. We do not have immediate hypotheses to explain these effects of *S. cerevisiae*, or why we saw the effect in flies inoculated with *S. cerevisiae.* Because this variation does not appear to affect desiccation survival, we do not explore it further here.

Larval nutrition can have far-reaching effects on adult performance (e.g. Vijendravarma et al., 2010). Competition for dietary yeasts mediates the effects of larval crowding on a range of traits, including structural body size (Klepsatel et al., 2018), which is an important determinant of desiccation survival in *Drosophila* (Gibbs and Matzkin, 2001). We found that flies reared with live *S. cerevisiae* and *L. kluyveri* survived desiccation for less time than their axenic counterparts, and we showed that the yeasts had to be alive to have this effect. Furthermore, we accounted for body size in our analyses, which suggests that the effects we see are not simply a consequence of larval food resources yielding different-sized flies with corresponding variation in capacity to maintain water balance. Thus, we conclude that the effects of yeasts we observed on adult flies were not mediated by variation in yeast nutritional availability during larval development.

Desiccation survival in *Drosophila* is not usually associated with changes in desiccation tolerance (i.e. water content at death; Gibbs, 2002; Gibbs et al., 1997; Gibbs et al., 2003; Gibbs and Matzkin, 2001), so it is surprising to see that some rearing conditions modify water content at death in adult flies. One possible explanation for this effect is that the live yeasts in the gut results in a net increase in whole body water content, but that the increased bulk water in the yeast cells is not available to the fly. Alternately, the increased body water content could lead to adult flies that do not regulate water balance as tightly, leading to lower survival when exposed to desiccating conditions. While water loss rates can be plastic (e.g. Bazinet et al., 2010) and could yield this result, we are not aware of corresponding plasticity in the amount of water loss tolerated in *Drosophila*. Relatively little is known about the cellular mechanisms causing death from dehydration in insects (Bradley, 2009; Gibbs et al., 1997; Hadley, 1994; Harrison et al.,

2012), which prevents us from speculating about the mechanistic link between gut yeast and desiccation tolerance in our experiments. However, we do not see equivalent effects of rearing with dead yeasts on water balance parameters or desiccation survival, which leads us to conclude that the impact of gut yeasts on water balance is dependent on the yeasts being alive, and either due to modification of digestive processes in the gut (cf. Wong et al., 2014), or a direct biological interaction between the yeast and its host (cf. Broderick et al., 2014; Schretter et al., 2018).

The effects of yeast rearing on both desiccation survival and individual water balance parameters in adult *D. melanogaster* appear to be species-specific. *Pichia kluyveri* is found in *D. melanogaster* guts in nature, and although it does not improve performance in our experiments, it is the only species we tested that was not in some way detrimental to desiccation survival, supporting the hypothesis that there is some degree of yeast-host coevolution, as suggested by Starmer and Fogleman (1986). Our results were largely consistent across cohorts for *P. kluyveri* and *L. kluyveri*, with the one exception being in female adults where inoculation with *P. kluyveri* was detrimental to desiccation survival in one cohort, but not the other. However, as our prediction was that yeast would improve desiccation survival, this does not modify our conclusion that gut yeasts do not improve desiccation survival. Rearing with *P. kluyveri* still increased both initial water content and water content at death in adult flies, which we suggest provides support for our contention that the increased water content we measured is not physiologically available to the fly. This hypothesis could be tested in the future using stable-isotope labelled water in the yeasts (cf. McCluney and Sabo, 2010). *Lachancea kluyveri* has not been collected from *D. melanogaster* in nature (although it is found in other *Drosophila* species),

and has positive impacts on both development time and chill coma recovery (Jiménez Padilla, 2016). Thus, the identification of improved performance in one or two traits (development time and chill coma) does not necessarily imply that the same yeast is beneficial for all traits (desiccation tolerance in this case). This implies that the choice of traits for studying host-microbe interactions is important when extrapolating to fitness or field performance.

In nature (and indeed, under normal circumstances in the laboratory), *Drosophila* larvae grow in a microbe-rich environment including abundant yeasts of many species, alongside bacteria and other microbes (Broderick and Lemaitre, 2012; Chandler et al., 2012; Wong et al., 2011). Our single-yeast gnotobiotic system is clearly unnatural (and we cannot rule out more complex interactions among diverse gut microbiota). It nonetheless allows us to demonstrate that gut yeasts can have an effect, and that the identity of those yeasts appears to be important in determining host performance (cf. Ben Ami et al., 2010). Although we report what appear to be negative (or at best neutral) consequences of yeast-fly coexistence, there appears to be coevolution in the wild (Starmer and Fogleman, 1986), and some of the same yeasts that have negative consequences for water balance in this study have positive impacts on other traits (Jiménez Padilla, 2016). Indeed, flies in the wild seek out yeast preferentially (Becher et al., 2012; Dobzhansky et al., 1956). We propose two non-mutually exclusive explanations for this effect. First, the effects of gut microbes can be context-dependent, for example conventional flies develop faster and store less lipid than axenic flies only in high-glucose, low-yeast diets (Wong et al., 2014). Thus, it is possible that yeasts are beneficial – even to water balance – in a field context that is not adequately captured in our laboratory experiments. Second, the water balance consequence of larval development with yeast may be inconsequential compared to the benefits

accrued to development time and thermal performance (Jiménez Padilla, 2016), such that yeast-host interactions are maintained.

Baker's yeast, *Saccharomyces cerevisiae*, is a standard ingredient in most *Drosophila* rearing media (Ashburner et al., 2005; Markow and O'Grady, 2005), even though it is seldom collected from *Drosophila* in nature (Hoang et al., 2015). We found that inoculation with *S. cerevisiae* had no impact on desiccation survival or water balance parameters. By contrast, larval rearing with *S. cerevisiae* decreased adult desiccation survival, increased initial water content, and slightly decreased water loss rate. These observations have two important implications. First, 'conventional' rearing with *S. cerevisiae* may have impacts that are not reflected in the biology of *Drosophila* in the field that primarily interact with other species of yeast. However, we do note that in our laboratory, where our conventionally-reared flies receive live *S. cerevisiae*, the predominant culturable yeast species from the vials is an as-yet unidentified species (Jiménez-Padilla and Lachance, unpublished observations). Thus, the overall microbiological *milieu* of human-built environments may be providing some inadvertent correction for our dependence on *S. cerevisiae* in *Drosophila* cultures, although we have not explored the yeast community of *Drosophila* cultures beyond our own laboratory to see if this is a widespread effect. Second, because it has phenotypic effects different to other yeasts, and is not normally associated with *D. melanogaster* in nature, *S. cerevisiae* may be an inappropriate yeast for experiments on gut yeast in insects (at least without direct comparison to other species of yeast more closely associated with the host; see also Hoang et al., 2015).

In conclusion, gut yeasts during development can affect water balance of adult *D. melanogaster*, but generally this effect is detrimental, appears to require long-term exposure to the yeasts during larval development, and depends on the yeasts being alive. There are among-species differences in the phenotypic effect of gut yeasts on water balance which are broadly consistent with some degree of co-evolution between yeast species and their insect hosts. Finally, we support the assertions of Hoang et al. (2015) that *S. cerevisiae* may not be the most appropriate species of yeast to use when examining the effects of gut yeasts on *Drosophila* biology.

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Figure Captions

Figure 1 – Survival of adult *Drosophila melanogaster* under desiccating conditions when axenic or inoculated as adults with three yeast species, (A) *Pichia kluyveri*, (B) *Lachancea kluyveri*, and (C) *Saccharomyces cerevisiae*. Mean (±SD) survival time; Males: dashed lines, square symbols, Females: solid lines, diamond symbols. There were no significant differences between axenic and gnotobiotic flies, and no sex × treatment interactions; † indicates significant differences between sexes ($P<0.05$). Sample sizes: *P. kluyveri* - 33 females, 26 males/treatment; *L. kluyveri* - 62 females, 61 males/treatment; *S. cerevisiae* 33 females, 32 males/treatment. See Table 1 for results of statistical analyses.

Figure 2 – Effect of yeast inoculation as adults on water balance parameters of *Drosophila melanogaster* under desiccating conditions. Mean ± SD shown for male (dashed lines, square symbols) and female (solid lines, diamonds) inoculated with *Pichia kluyveri* (A,B,C), *Lachancea kluyveri* (D,E,F), or *Saccharomyces cerevisiae* (G,H,I). There were no significant sex × treatment interactions. * and † indicate significant differences between gnotobiotic and axenic-treated flies, and between sexes, respectively ($P<0.05$). Statistics in Table 1; these parameters are separated by cohort in Fig. S6.

Figure 3 – Survival of adult *Drosophila melanogaster* under desiccating conditions after rearing under axenic conditions or with three yeast species, (A, D) *Pichia kluyveri*, (B, E) *Lachancea kluyveri*, and (C, F) *Saccharomyces cerevisiae* which are alive (A, B, C) or heat-killed (D, E, F). Mean (±SD) survival time; Males: dashed lines, square symbols, Females: solid lines, diamond

symbols. There were no significant sex × treatment interactions; * indicates significant difference between axenic and gnotobiotic flies, † indicates significant differences between sexes ($P<0.05$). Live yeasts sample sizes: *P. kluyveri* - 27 females, 32 males/treatment; *L. kluyveri* - 31 females, 34 males/treatment; *S. cerevisiae* 31 females, 32 males/treatment. Dead yeast sample sizes: *P. kluyveri* - 23 females, 26 males/treatment; *L. kluyveri* - 24 females, 24 males/treatment; *S. cerevisiae* 25 females, 24 males. See Tables 2 and 3 for statistics.

Figure 4 – Effect of rearing with live yeast on water balance parameters of adult *Drosophila melanogaster* under desiccating conditions. Mean ± SD shown for male (dashed lines, square symbols) and female (solid lines, diamonds) inoculated with *Pichia kluyveri* (A,B,C), *Lachancea kluyveri* (D,E,F), or *Saccharomyces cerevisiae* (G,H,I). Sex × treatment interactions were significant for initial water content for *P. kluyveri* and water loss rate for *S. cerevisiae*. * and † indicate significant differences between gnotobiotic and axenic-treated flies, and between sexes, respectively ($P<0.05$). Statistics in Table 2.

Figure 5 - Effect of rearing with heat-killed yeasts on water balance parameters of adult *Drosophila melanogaster* under desiccating conditions. Mean ± SD shown for male (dashed lines, square symbols) and female (solid lines, diamonds) inoculated with *Pichia kluyveri* (A,B,C), *Lachancea kluyveri* (D,E,F), or *Saccharomyces cerevisiae* (G,H,I). There were no significant sex × treatment interactions . * and † indicate significant differences between gnotobiotic and axenic-treated flies, and between sexes, respectively; Statistics in Table 3.

Table 1 - Summary of Analysis of Covariance of water balance and desiccation survival of axenic and gnotobiotic *D. melanogaster* inoculated with *Pichia kluyveri*, *Lachancea kluyveri*, and *Saccharomyces cerevisiae*. Cohorts are separated according to date of the experiment (all *S. cerevisiae* experiments were conducted on the same day, so there is no cohort term in the model); significant terms are in bold typeface; all p-values have been subjected to a study-wide Benjamini-Hochberg FDR correction.

Initial Water Content			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,123}$=13.5, P<0.01**	**$F_{1,205}$=165.1, P<0.01**	**$F_{1,118}$=44.2, P<0.01**
Cohort	$F_{1,122}$=0.3, P=0.67	$F_{1,203}$=0.1, P=0.84	-
Dry Mass	**$F_{1,123}$=14.0, P<0.01**	**$F_{1,205}$=107.8, P<0.01**	**$F_{1,118}$=79.8, P<0.01**
Treatment	$F_{1,121}$=0.3, P=0.43	$F_{1,204}$=1.6, P=0.30	$F_{1,118}$=3.9, P=0.10
Sex × Treatment	$F_{1,120}$=0.5, P=0.61	$F_{1,202}$=0.1, P=0.79	$F_{1,117}$=0.4, P=0.62
Water Content at death			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	$F_{1,122}$=1.9, P=0.26	**$F_{1,204}$=13.8, P<0.01**	**$F_{1,120}$=27.7, P<0.01**
Cohort	**$F_{1,123}$=47.7, P<0.01**	**$F_{1,204}$=162.1, P<0.01**	-
Dry Mass	**$F_{1,123}$=29.1, P<0.01**	**$F_{1,204}$=13.8, P<0.01**	$F_{1,119}$=3.9, P=0.62
Treatment	$F_{1,121}$=1.1, P=0.44	$F_{1,203}$=3.3, P=0.12	$F_{1,118}$=0.9, P=0.48
Sex × Treatment	$F_{1,120}$=2.3, P=0.20	$F_{1,202}$=0.9, P=0.46	$F_{1,117}$=3.8, P=0.11
Water Loss Rate			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	$F_{1,124}$=1.7, P=0.30	**$F_{1,204}$=11.4, P<0.01**	$F_{1,118}$=0.04, P=0.88
Cohort	$F_{1,122}$=0.3, P=0.70	**$F_{1,204}$=144.4, P<0.01**	-
Dry Mass	$F_{1,121}$=0.3, P=0.67	$F_{1,204}$=4.8, P=0.06	**$F_{1,119}$=6.4 P=0.02**
Treatment	$F_{1,123}$=0.4, P=0.62	$F_{1,203}$=3.0, P=0.14	$F_{1,119}$=4.3, P=0.08
Sex × Treatment	$F_{1,120}$=1.3, P=0.37	$F_{1,202}$=0.6, P=0.58	$F_{1,117}$=0.0001, P=0.99
Survival time under desiccating conditions			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,122}$=15.9, P<0.01**	**$F_{1,204}$=31.8, P<0.01**	**$F_{1,119}$=26.6, P<0.001**
Cohort	**$F_{1,122}$=8.4, P=0.01**	**$F_{1,204}$=50.7, P<0.01**	-
Dry Mass	$F_{1,122}$=4.1, P=0.10	**$F_{1,204}$=14.8, P<0.01**	**$F_{1,119}$=22.4, P<0.01**
Treatment	$F_{1,121}$=2.4, P=0.20	$F_{1,203}$=0.3, P=0.68	$F_{1,118}$=0.02, P=0.93
Sex × Treatment	$F_{1,120}$=2.3, P=0.20	$F_{1,202}$=0.0001, P=0.99	$F_{1,117}$=0.6, P=0.56

Table 2- Summary of Analysis of Covariance of water balance and desiccation survival of axenic and gnotobiotic *D. melanogaster* reared with *Pichia kluyveri*, *Lachancea kluyveri*, and *Saccharomyces cerevisiae*. Significant terms are in bold typeface; all p-values have been subjected to a study-wide Benjamini-Hochberg FDR correction.

Initial Water Content			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,115}$=116.4, P<0.01**	**$F_{1,120}$=89.9, P<0.01**	**$F_{1,120}$=32.5, P<0.01**
Dry Mass	$F_{1,114}$=1.6, P=0.33	**$F_{1,120}$=13.8, P<0.01**	$F_{1,119}$=0.4, P=0.62
Treatment	**$F_{1,115}$=48.3, P<0.01**	**$F_{1,120}$=154.5, P<0.01**	**$F_{1,120}$=61.0, P<0.01**
Sex × Treatment	**$F_{1,115}$=6.9, P=0.02**	$F_{1,120}$=7.9, P=0.11	$F_{1,118}$=0.1, P=0.80
Water Content at death			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,115}$=46.7, P<0.01**	**$F_{1,121}$=12.8, P<0.01**	**$F_{1,121}$=25.6, P<0.01**
Dry Mass	$F_{1,114}$=0.1, P=0.80	**$F_{1,121}$=7.4, P=0.02**	$F_{1,120}$=2.9, P=0.16
Treatment	**$F_{1,115}$=15.5, P<0.01**	**$F_{1,121}$=35.4, P<0.01**	$F_{1,119}$=1.4, P=0.35
Sex × Treatment	$F_{1,115}$=5.0, P=0.06	$F_{1,120}$=0.003, P=0.97	$F_{1,118}$=0.7, P=0.55
Water Loss Rate			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	$F_{1,115}$=0.4, P=0.62	$F_{1,121}$=2.5, P=0.20	**$F_{1,118}$=9.5, P<0.01**
Dry Mass	$F_{1,116}$=0.5, P=0.60	$F_{1,122}$=0.4, P=0.65	$F_{1,118}$=4.0, P=0.10
Treatment	$F_{1,117}$=3.4, P=0.12	**$F_{1,123}$=75.1, P<0.01**	**$F_{1,118}$=68.8, P<0.01**
Sex × Treatment	$F_{1,114}$=1.4, P=0.36	$F_{1,120}$=2.3, P=0.20	**$F_{1,118}$=11.6, P<0.01**
Survival time under desiccating conditions			
Effect	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,117}$=41.0, P<0.01**	**$F_{1,122}$=54.1, P<0.01**	**$F_{1,120}$=54.9, P<0.01**
Dry Mass	$F_{1,115}$=0.2, P=0.73	$F_{1,121}$=0.007, P=0.97	$F_{1,119}$=0.1, P=0.82
Treatment	$F_{1,116}$=0.2, P=0.78	**$F_{1,122}$=64.8, P<0.01**	**$F_{1,120}$=37.3, P<0.01**
Sex × Treatment	$F_{1,114}$=0.8, P=0.49	$F_{1,120}$=0.7, P=0.56	$F_{1,118}$=0.9, P=0.49

Table 3 - Summary of Analysis of Covariance (ANCOVA) of water balance and desiccation survival of axenic and gnotobiotic *D. melanogaster* reared with heat-killed *Pichia kluyveri*, *Lachancea kluyveri*, and *Saccharomyces cerevisiae*. Significant terms are in bold typeface; all p-values have been subjected to a study-wide Benjamini-Hochberg FDR correction.

Initial Water Content			
Effects	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,92}$=38.6 P<0.01**	**$F_{1,92}$=41.8, P<0.01**	**$F_{1,93}$=207.7, P<0.01**
Dry Mass	**$F_{1,92}$=230.5, P<0.01**	**$F_{1,92}$=69.7, P<0.01**	**$F_{1,92}$=53.8, P<0.01**
Treatment	$F_{1,91}$=0.13, P=0.12	$F_{1,91}$=3.5, P=0.11	**$F_{1,92}$=9.1, P<0.01**
Sex × Treatment	$F_{1,91}$=0.005, P=0.97	$F_{1,90}$=0.003, P=0.97	$F_{1,91}$=2.8, P=0.17
Water Content at death			
Effects	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	$F_{1,92}$=4.9, P=0.06	**$F_{1,93}$=5.6, P=0.04**	$F_{1,92}$=2.3, P=0.20
Dry Mass	**$F_{1,92}$=10.4, P<0.01**	$F_{1,92}$=2.7, P=0.19	**$F_{1,93}$=23.7, P<0.01**
Treatment	**$F_{1,92}$=26.4, P<0.01**	$F_{1,91}$=1.5, P=0.33	**$F_{1,93}$=39.2, P<0.01**
Sex × Treatment	$F_{1,91}$=2.1, P=0.23	$F_{1,90}$=3.3, P=0.12	$F_{1,91}$=0.5, P=0.62
Water Loss Rate			
Effects	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,92}$=11.1, P<0.01**	**$F_{1,93}$=6.8, P=0.02**	**$F_{1,93}$=16.8, P<0.01**
Dry Mass	**$F_{1,92}$=9.4, P<0.01**	$F_{1,92}$=0.2, P=0.73	$F_{1,92}$=3.4, P=0.12
Treatment	**$F_{1,92}$=15.2, P<0.01**	$F_{1,91}$=0.1, P=0.86	**$F_{1,93}$=16.8, P=0.04**
Sex × Treatment	$F_{1,91}$=0.6, P=0.57	$F_{1,90}$=0.004, P=0.97	$F_{1,91}$=0.7, P=0.54
Survival time under desiccating conditions			
Effects	***Pichia kluyveri***	***Lachancea kluyveri***	***Saccharomyces cerevisiae***
Sex	**$F_{1,93}$=71.4, P<0.01**	**$F_{1,92}$=54.1, P<0.01**	**$F_{1,92}$=5.4, P=0.04**
Dry Mass	**$F_{1,93}$=36.0, P<0.01**	**$F_{1,92}$=23.8, P<0.01**	**$F_{1,92}$=104.6, P<0.01**
Treatment	$F_{1,92}$=3.4, P=0.12	$F_{1,91}$=2.3, P=0.20	**$F_{1,92}$=5.8, P=0.04**
Sex × Treatment	$F_{1,91}$=0.1, P=0.79	$F_{1,90}$=0.5, P=0.62	$F_{1,91}$=0.3, P=0.67

567 **Figure 1**

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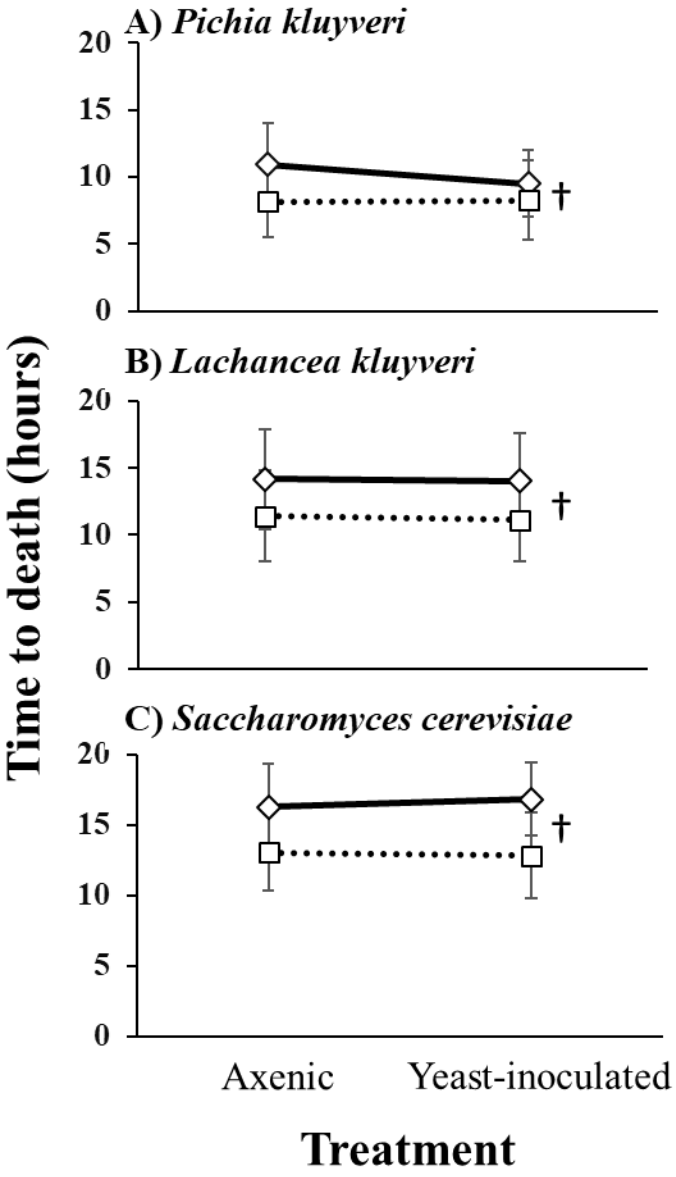

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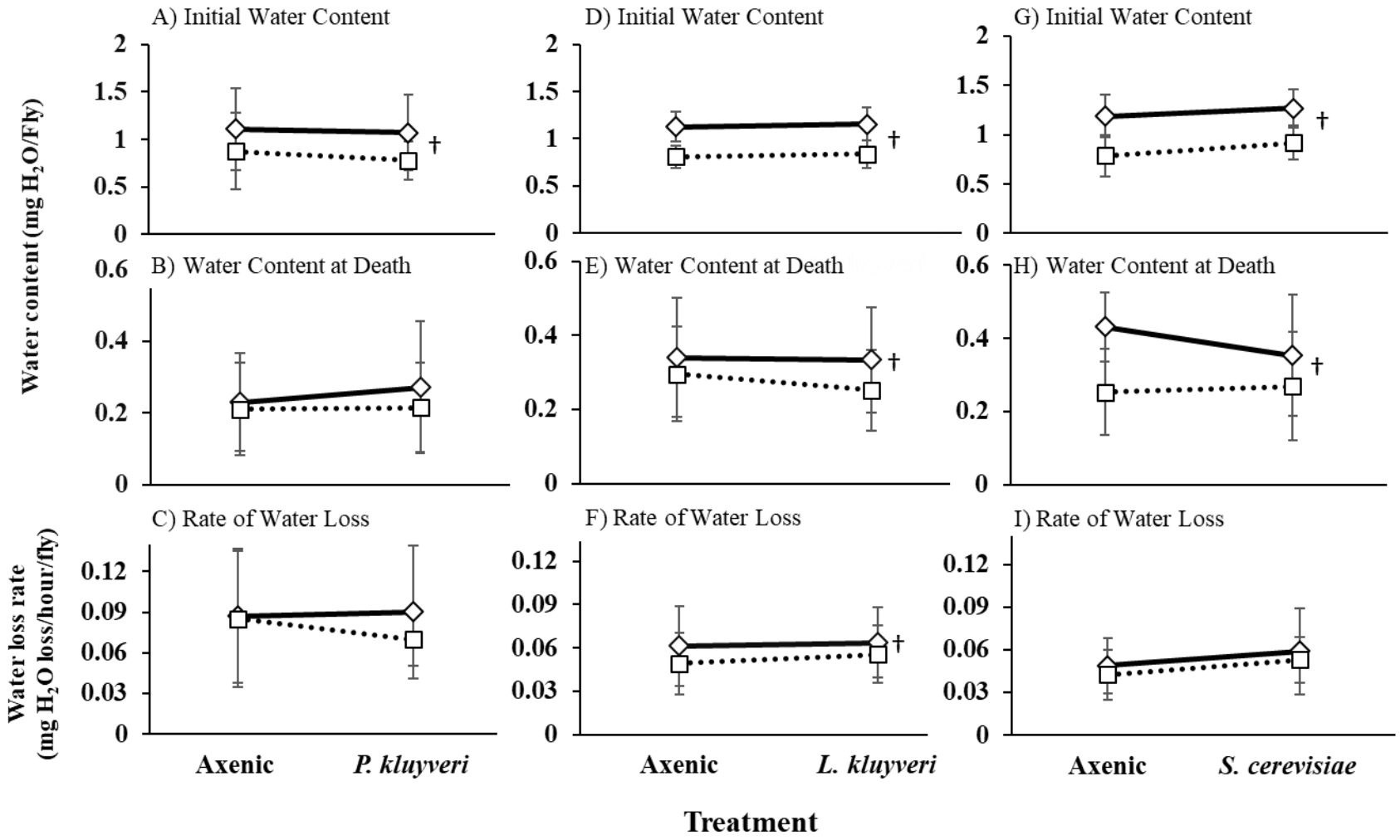
571 Figure 2



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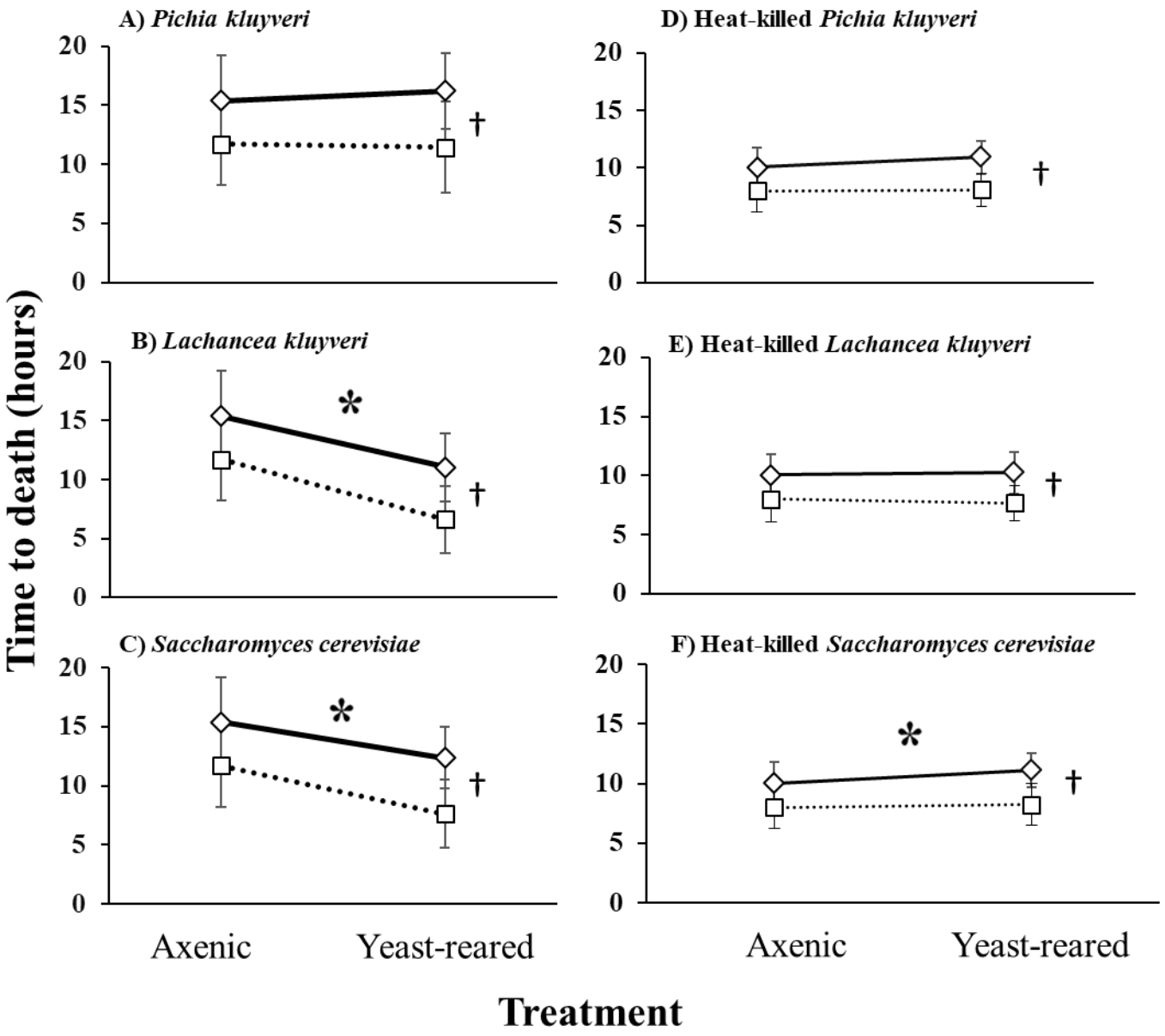
574 Figure 3



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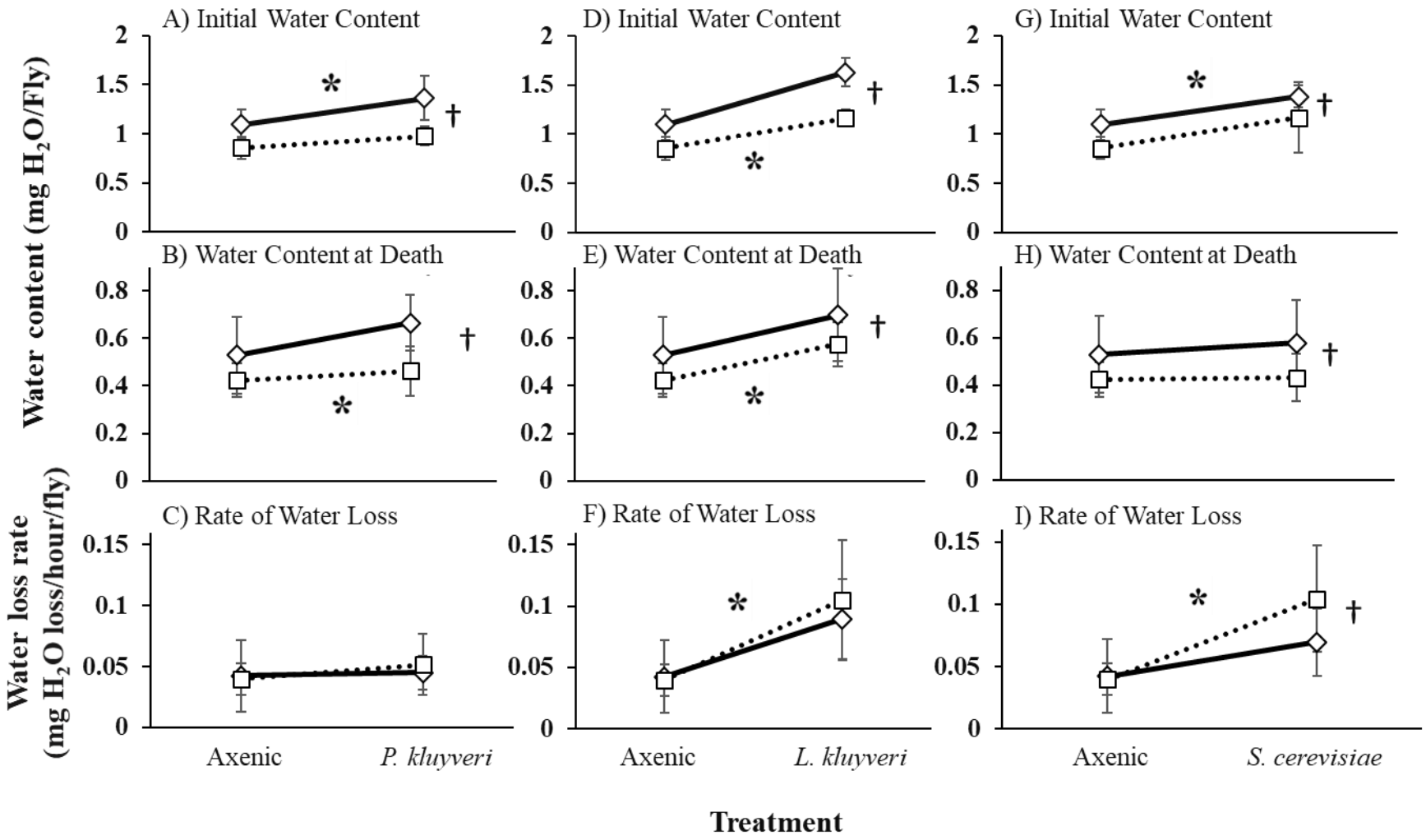
577 Figure 4



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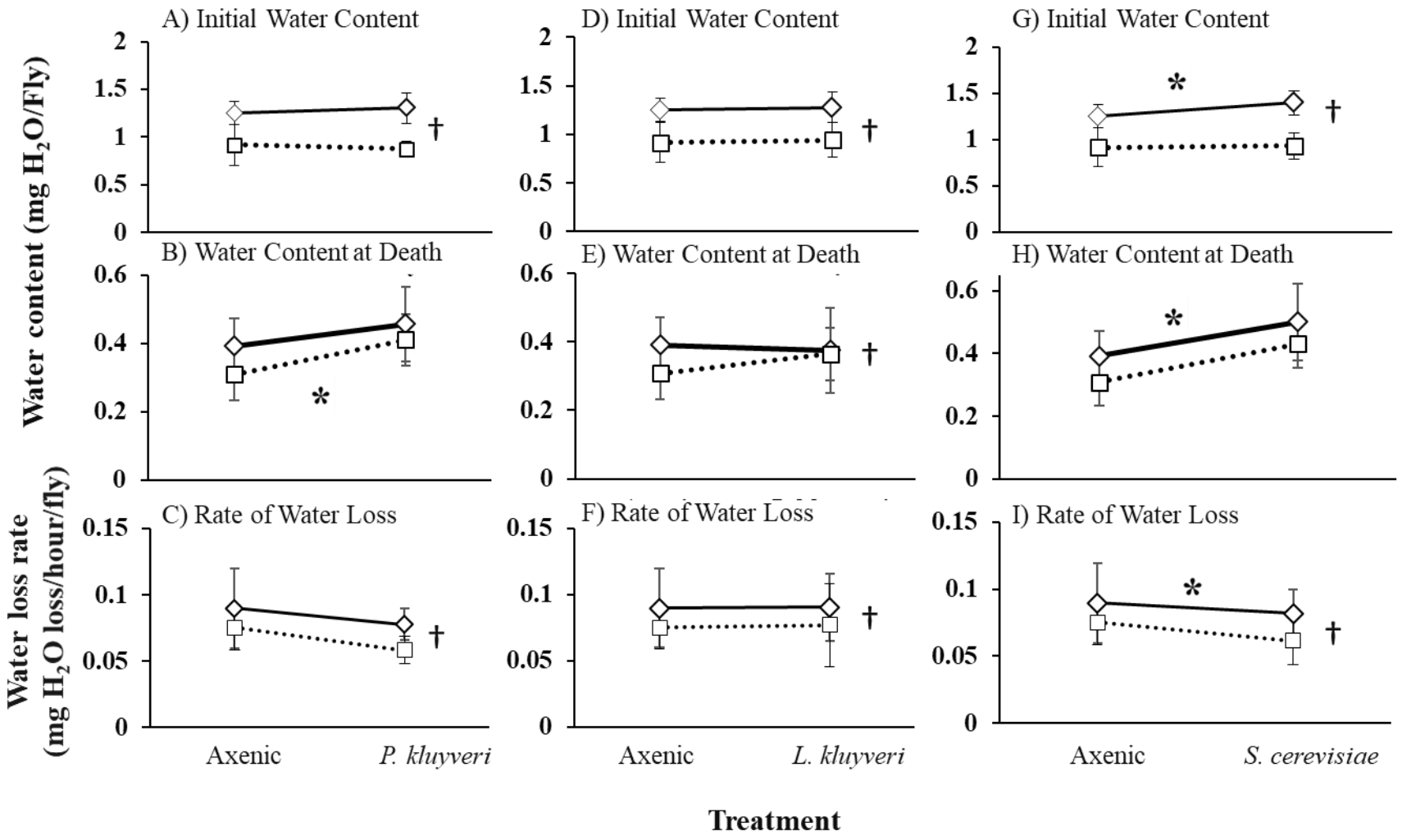
581 Figure 5



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