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**Storage length and temperature influence infectivity  
and spore yield of two common *Daphnia* parasites**

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Running head: Effects of storage on *Daphnia* parasites

Keywords: *Metschnikowia*; *Pasteuria*; refrigerator; freezer; burst size; spore; fitness; *Daphnia dentifera*

## 22 **Abstract**

23 *Daphnia* and their parasites have emerged as a model system for understanding the  
24 ecology and evolution of infectious diseases. Two of the most commonly studied *Daphnia*  
25 parasites are the bacterium *Pasteuria ramosa* and the fungus *Metschnikowia bicuspidata*. In  
26 addition to being the focus of numerous field studies, these two parasites have been used in many  
27 laboratory experiments. However, there is little information in the scientific literature about how  
28 the conditions under which these parasites are stored influence the infectivity and yield of  
29 transmission stages (“spores”). This is problematic because such information is critical for  
30 experiment design and data interpretation.

31 We tested the influence of storage length (eight treatments ranging from 1 day to 1 year)  
32 and temperature (-20°C (freezer) vs. 4°C (refrigerator)) on spore infectivity and yield. We found  
33 that *Pasteuria* spores survived well at both -20°C and 4°C, and remained infective even after  
34 storage for one year. However, *Pasteuria* spore yields dropped over time, particularly at 4°C. In  
35 contrast, *Metschnikowia* spores were killed within days at -20°C. At 4°C, *Metschnikowia*  
36 infectivity declined steadily over a period of two months and, by four months, spores were no  
37 longer infective. Spore yield from *Metschnikowia*-infected hosts was not significantly impacted  
38 by storage length, but trended downwards.

39 Scientists working with *Pasteuria* should be aware that spore yield declines during  
40 storage, particularly in the refrigerator. Scientists working with *Metschnikowia* should be aware  
41 that it is killed by freezer storage and that, even if it is stored in the refrigerator, infectivity  
42 declines within a few months. These results might have implications for parasite distributions in  
43 the field; for example, the high sensitivity of *Metschnikowia* to freezing might help explain why  
44 it tends to be more common in deep lakes than in ponds or rock pools.

## 45 **Introduction**

46           Studies of *Daphnia*–parasite interactions date back to at least the 1880s (Metchnikoff  
47 1884, 1888, 1889). In the past 25 years, *Daphnia* and their parasites have emerged as a model  
48 system for understanding the ecology and evolution of infectious diseases (Ebert 2005, Lampert  
49 2011, Cáceres et al. 2014). One strength of the *Daphnia*–parasite system is its utility in  
50 laboratory experiments, which have helped uncover factors that are relevant to the ecology and  
51 evolution of infectious diseases (Ebert 2008, Ebert 2011, Lampert 2011).

52           Despite being an important study system, there is surprisingly little information in the  
53 scientific literature about how lab conditions, including storage length and temperature, influence  
54 transmission stages of common *Daphnia* parasites. These transmission stages (hereafter:  
55 “spores”) are the infective stage of the parasite, enabling the parasite to move between host  
56 individuals. If lab storage conditions influence the infectivity and/or yield of parasite spores, that  
57 would affect the likelihood of an experiment succeeding. Moreover, since scientists often use  
58 infectivity and spore yield as proxies for parasite fitness, impacts of lab storage on spores could  
59 also affect estimates of parasite fitness.

60           Thus, understanding whether lab conditions alter spore infectivity and yield is critical for  
61 experimental design. For example, we recently carried out an experiment in which we collected  
62 spores of the common bacterial parasite, *Pasteuria ramosa*, from multiple lakes and had to store  
63 them in the lab for a year before using them in experiments. We knew that *Pasteuria* spores  
64 could remain viable in the lab for this length of time (Ebert 2005, Little et al. 2006), but had no  
65 information on whether—to maximize future infectivity and spore yield—it would be better to  
66 store them in the refrigerator (-4°C) or freezer (-20°C). For a second experiment, we wanted to  
67 kill spores of the common fungal parasite, *Metschnikowia bicuspidata*, without killing *Pasteuria*

68 spores. We knew that *Pasteuria* spores remain viable in the freezer (Ebert 2005, Little et al.  
69 2006), even after passing through a *Daphnia* gut (King et al. 2013). However, we did not have  
70 corresponding data on *Metschnikowia*, though, based on anecdotal observations, we suspected  
71 *Metschnikowia* spores would not remain viable in the freezer. Our two studies showed that we  
72 need more information regarding spore storage conditions to make informed decisions about  
73 appropriate experimental design.

74 We tested the effects of storage length and temperature on two common *Daphnia*  
75 parasites: *Pasteuria ramosa* and *Metschnikowia bicuspidata*. We stored spores for lengths  
76 ranging from 1 day to 1 year and at two temperatures (-20°C (freezer) and 4°C (refrigerator)),  
77 then assessed both their infectivity and the spore yield from infected hosts. This information will  
78 assist other scientists interested in carrying out experiments with these model systems, and might  
79 also help explain patterns of disease in natural populations.

80

## 81 **Methods**

82 We tested the effects of storage length and temperature on infectivity of spores of the  
83 bacterium *Pasteuria ramosa* (“G/18” isolate) and the fungus *Metschnikowia bicuspidata* (“Std”  
84 isolate). We used infection assays in which hosts were exposed to either *Pasteuria* or  
85 *Metschnikowia*. While carrying out the experiment, one of us (KKH) noticed that the number of  
86 spores per tube seemed to decrease over time. Thus, we also did a post-hoc analysis to determine  
87 whether spore yield varied with storage length and/or temperature.

88 Our general procedure involved: 1) collecting *D. dentifera* that were heavily infected  
89 with either *Pasteuria* or *Metschnikowia* and putting them in microcentrifuge tubes (6–9  
90 individuals per tube); 2) assigning each tube to a particular storage length x storage temperature

91 treatment; 3) after the appropriate storage length, removing them from storage and grinding up  
92 the infected individuals in each tube; 4) using a hemocytometer to determine the density of  
93 spores in each tube (our measure of spore yield); 5) adding the appropriate amount of spores  
94 from a tube to a beaker containing six uninfected *D. dentifera*; 6) exposing the *D. dentifera* to the  
95 spores for 24 hours; and, finally, 7) maintaining the exposed individuals in the lab until it was  
96 possible to determine whether they were infected (our measure of infectivity).

97

98 *(a) Impact of storage length and temperature on spore infectivity*

99 We used eight storage length treatments, ranging from 1 day to 1 year (Table 1). We used  
100 this range because sometimes we want to store spores longer term (as in the *Pasteuria* example  
101 given in the introduction), whereas at other times we want to quickly kill spores (as in the  
102 *Metschnikowia* example given in the introduction). The intermediate storage ranges were chosen  
103 because, based on prior experience with these parasites, we suspected that *Pasteuria* would live  
104 at least several months and likely a full year, whereas we suspected that *Metschnikowia* would  
105 not survive more than a few months. We used two storage temperatures (-20°C vs. 4°C), chosen  
106 to correspond with common laboratory storage conditions: in the freezer or refrigerator,  
107 respectively.

108

109 **Table 1.** Treatment combinations for the experiment. “*Pasteuria*” = *Pasteuria ramosa*; “*Metsch*” = *Metschnikowia bicuspidata*; “wks”  
 110 = weeks, “mo.” = months. For some of the *Pasteuria* treatments, there were not enough spores to follow the initial plan of two low  
 111 spore beakers and two high spore beakers per replicate tube. Full details can be found in the online repository for the data and code.

Parasite	Storage temp. (°C)	Storage length	Spore doses (spores/mL)	# infected individuals per tube	# replicate tubes	# beakers per replicate tube		Date of exposure (month/day/year)
						Low spore dose	High spore dose	
<i>Pasteuria</i>	4	1 day	500, 2000	6	5	2	2	3/14/2017
<i>Pasteuria</i>	4	2 wks	500, 2000	6	5	2	2	3/27/2017
<i>Pasteuria</i>	4	4 wks	500, 2000	6	5	1-3	2	4/10/2017
<i>Pasteuria</i>	4	2 mo.	500, 2000	6	5	2	2	5/30/2017
<i>Pasteuria</i>	4	4 mo.	500, 2000	6	5	2	2	7/11/2017
<i>Pasteuria</i>	4	6 mo.	500, 2000	6	4-5	2-4	1-2	9/18/2017
<i>Pasteuria</i>	4	9 mo.	500, 2000	6	4-5	2-4	1-2	12/11/2017
<i>Pasteuria</i>	4	1 year	500, 2000	6	4-5	2	1-2	3/6/2018
<i>Pasteuria</i>	-20	1 day	500, 2000	6	5	2	2	3/14/2017
<i>Pasteuria</i>	-20	2 wks	500, 2000	6	5	2	2	3/27/2017
<i>Pasteuria</i>	-20	4 wks	500, 2000	6	5	2	2	4/10/2017
<i>Pasteuria</i>	-20	2 mo.	500, 2000	6	5	2	2	5/30/2017
<i>Pasteuria</i>	-20	4 mo.	500, 2000	6	5	2	2	7/11/2017
<i>Pasteuria</i>	-20	6 mo.	500, 2000	6	5	2	2	9/18/2017
<i>Pasteuria</i>	-20	9 mo.	500, 2000	6	4-5	2	2-4	12/11/2017
<i>Pasteuria</i>	-20	1 year	500, 2000	6	5	2	2	3/6/2018
<i>Metsch.</i>	4	1 day	250, 1000	9	3	2	2	3/14/2017
<i>Metsch.</i>	4	2 wks	250, 1000	9	3	2	2	3/27/2017
<i>Metsch.</i>	4	4 wks	250, 1000	9	3	2	2	4/10/2017
<i>Metsch.</i>	4	2 mo.	250, 1000	9	3	2	2	5/30/2017
<i>Metsch.</i>	4	4 mo.	250, 1000	9	3	2	2	7/11/2017
<i>Metsch.</i>	4	6 mo.	250, 1000	9	3	2	2	9/18/2017
<i>Metsch.</i>	4	9 mo.	250, 1000	9	3	2	2	12/11/2017
<i>Metsch.</i>	4	1 year	250, 1000	9	3	2	2	3/6/2018
<i>Metsch.</i>	-20	1 day	1000	7	3	0	2	3/14/2017
<i>Metsch.</i>	-20	2 wks	1000	7	3	0	2	3/27/2017

112

113 We maintain separate laboratory stock cultures of *Pasteuria* and *Metschnikowia*. We  
114 collected heavily infected *Daphnia dentifera* from these cultures on 13 March 2017; individuals  
115 were identified as heavily infected based on their increased opacity, which makes them appear  
116 bright when illuminated. We put *Pasteuria*-infected *D. dentifera* in 1.5 mL microcentrifuge tubes  
117 that were then randomly assigned to a particular temperature and storage length treatment. We  
118 then repeated the process for *Metschnikowia*-infected *D. dentifera*. The exact number of infected  
119 individuals that were added to each tube is given in Table 1.

120 For parasite exposures, we used two spore doses for each parasite, as we thought the  
121 lower dose would detect treatment effects at times when infectivity remained high, while the  
122 higher dose could detect differences between treatments when infectivity was low. Pilot studies  
123 indicated that *Metschnikowia* dies rapidly when stored at -20°C. Therefore, for the -20°C  
124 *Metschnikowia* treatment, we used only the high dose exposure as we expected to see no  
125 infections at the low spore dose, and included only the two shortest storage lengths, to avoid  
126 wasting spores.

127 We used a pestle (Fisherbrand Pellet Pestle with Cordless Motor) to grind infected  
128 *Daphnia* in one of the microcentrifuge tubes (corresponding to the treatments indicated in Table  
129 1) and used a hemocytometer to determine the spore density in the tube. We then generated the  
130 desired spore dose by adding the appropriate volume from the tube (which contained 6–9  
131 infected individuals, as indicated in Table 1) to a 150 mL beaker filled with 100 mL of filtered  
132 lake water (*D. dentifera* survival is greatly enhanced by culturing in filtered lake water as  
133 compared to an artificial medium). Each beaker contained six uninfected *D. dentifera* that were  
134 six days old. We used the “Mid37” genotype, which is susceptible to both the “G/18” *Pasteuria*  
135 isolate and the “Std” *Metschnikowia* isolate (Auld et al. 2014a). The number of replicate tubes is

136 indicated in Table 1; with the exception of the *Metschnikowia* -20°C treatment (which did not  
137 have low spore beakers), a single 1.5 mL microcentrifuge tube was used to generate the spores  
138 for low spore dose and high spore dose beakers, as indicated in Table 1 in the “# beakers per  
139 replicate tube” columns.

140 We exposed *D. dentifera* to spores at 20°C in 16:8 light:dark (consistent with previous  
141 studies; e.g., Auld et al. 2014a). After 24 hours of spore exposure, the six *D. dentifera*  
142 individuals in each beaker were transferred to beakers containing filtered lake water that did not  
143 contain spores. Individuals were fed 10,000 cells/mL of a nutritious green alga, *Ankistrodesmus*  
144 *falcatus*, during parasite exposure and 20,000 cells/mL otherwise; we used a lower food dose  
145 during parasite exposure because it results in higher infection levels at a given spore dose (Hall  
146 et al. 2007). We added food to beakers four days/week; feeding four vs. seven days a week has  
147 no impact on our ability to determine whether individuals are infected, and reduces labor  
148 associated with feeding. Individuals were maintained in the lab until it was possible to determine  
149 whether they had been successfully infected (approximately 28 days for *Pasteuria* and 10-11  
150 days for *Metschnikowia*). Infections were diagnosed by observing individuals under a dissecting  
151 microscope; as indicated earlier, infected hosts are much more opaque, with spores filling their  
152 hemolymph.

153 We carried out two analyses of the infectivity data, both using generalized linear models  
154 (glms) with a binomial error structure. First, we analyzed the *Pasteuria* data using a model that  
155 included dose, storage temperature, storage length (in days), and their interactions as  
156 independent variables, and a matrix of uninfected and infected hosts (per individual beaker) as  
157 the response variable. For these analyses, we used storage length as measured in days, using the  
158 exact lengths calculated from the difference between 13 March and the day of exposure given in



159 Table 1. Second, we analyzed the data from the *Metschnikowia* 4°C treatment on its own, as we  
160 only had two storage lengths for the -20°C treatment. This model included dose, storage length  
161 (in days), and their interaction as independent variables, and a matrix of uninfected and infected  
162 hosts (per individual beaker) as the response variable. In some cases, the number of days did not  
163 precisely match to the treatment label (in months or years) due to logistical constraints (e.g., the  
164 “1 year” treatment was actually 358 days). The exact treatment lengths were as follows: 1 day (1  
165 day), 2 weeks (14 days), 4 weeks (28 days), 2 months (78 days), 4 months (120 days), 6 months  
166 (189 days), 9 months (273 days), 1 year (358 days).

167

#### 168 (b) Variation in spore yield over time

169 We also used glms to analyze the spore yield data, which was obtained with the  
170 hemocytometer counts described above. We analyzed data on *Pasteuria* spore yield using a  
171 model that included dose, storage length (in days), storage temperature, and their interactions.  
172 For the analysis of *Metschnikowia* spore yield in the 4°C treatment, we used a model that  
173 included dose, storage length (in days), and their interaction as independent variables. For both  
174 analyses, the dependent variable was ln(spores per infected *Daphnia*).

175 All analyses were done in R 3.5.0. All data and code can be found on github:

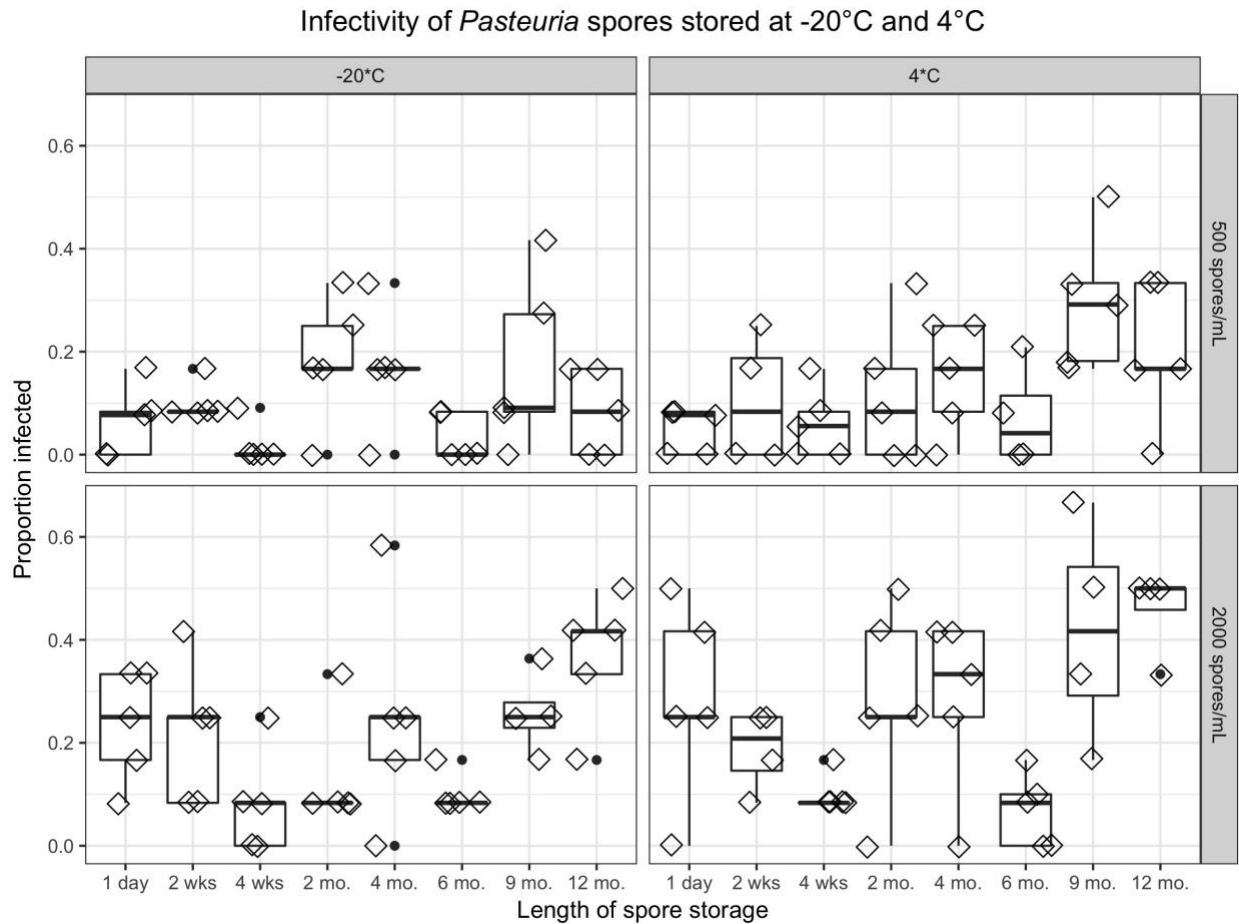
176 <https://github.com/duffymeg/sporestorage>.

177

## 178 Results

179 *Pasteuria* spores remained infective even after 1 year of storage (Figure 1; Table 2). Post-  
180 hoc analyses indicate that the increase in infectivity with storage length was significant for the  
181 high spore treatment at both temperatures, and for the low spore treatment at 4°C.

182



183

184 **Figure 1.** Spores of the bacterium *Pasteuria ramosa* remained infective even after a year in  
185 storage. Top row: Low spore dose treatment (500 spores/mL); bottom: high spore dose treatment  
186 (2000 spores/mL). Left: -20°C treatment (freezer); right: 4°C treatment (refrigerator). Spores  
187 remained infective in all of these treatments. Post-hoc analyses indicated that infectivity  
188 increased in the high spore treatment at both temperatures and in the low spore treatment at 4°C  
189 ( $p < 0.015$  in all three treatments).

190

191

192 **Table 2.** Results of statistical analysis of factors influencing infectivity of *Pasteuria* spores.

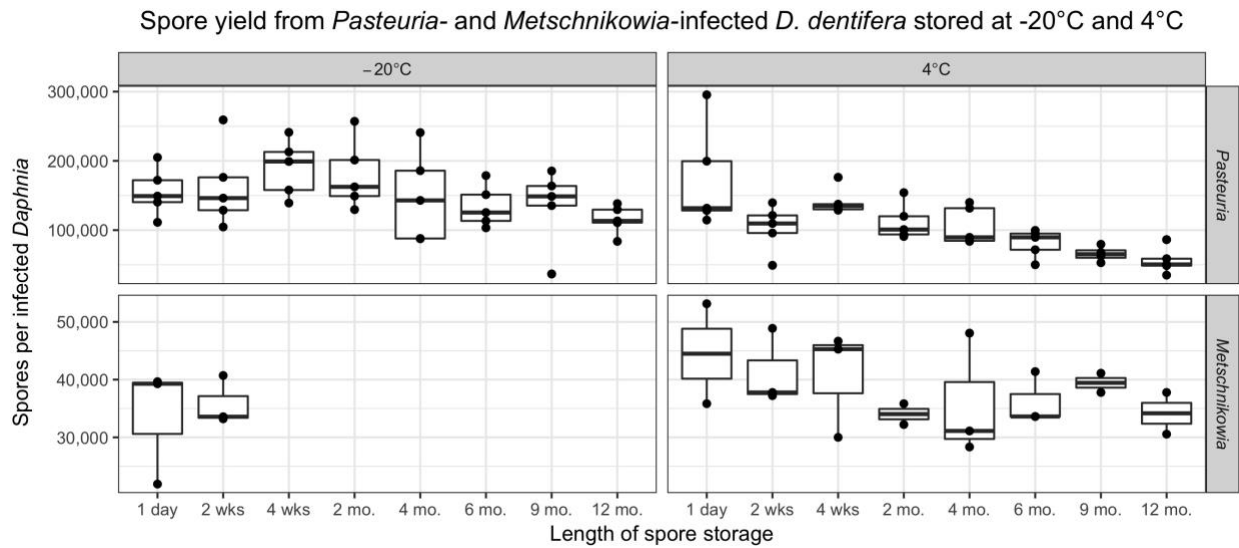
<b>Effect</b>	<b>Z</b>	<b>p</b>
Storage temperature	-0.63	0.529
Storage length	0.83	0.408
Dose	2.06	<b>0.040</b>
Storage temperature * storage length	2.01	<b>0.044</b>
Storage temperature * dose	1.10	0.273
Storage length * dose	0.93	0.353
Storage temperature * storage length * dose	-1.43	0.153

193

194 Spore yield from *Pasteuria*-infected hosts declined over time during the study, especially  
195 at 4°C (Figure 2; storage length:  $t = -2.84$ ,  $p = 0.006$ ; storage temperature:  $t = -2.04$ ,  $p = 0.045$ ;  
196 interaction:  $t = -2.50$ ,  $p = 0.014$ ). Even in the -20°C treatment, there was a significant drop in  
197 spore yield with increased storage length ( $t = -2.69$ ,  $p = 0.011$ ). It is possible that the increase in  
198 infectivity seen above (Figure 1) and the drop in spore yield (Figure 2) are both explained by  
199 non-viable spores degrading over time to the point where we could not visually identify them as  
200 spores.

201

202



203

204 **Figure 2.** Spore yield from *Pasteuria*-infected hosts (top row) declined with increasing storage

205 length, especially at 4°C (top right panel). Spore yield from *Metschnikowia*-infected hosts stored

206 at 4°C (bottom right panel) did not change significantly over time, but trended downwards.

207

208 Infectivity of *Metschnikowia* spores was strongly influenced by temperature and length of

209 storage (Figure 3). Contrary to *Pasteuria*, *Metschnikowia* spores died rapidly in the freezer. Only

210 one individual became infected out of the 36 that were exposed to a high dose of *Metschnikowia*

211 spores that had been stored at -20°C for 24 hours; in contrast, 17 of the 36 individuals exposed to

212 spores that had been stored at 4°C for 24 hours became infected. No individuals became infected

213 when exposed to *Metschnikowia* spores that had been stored at -20°C for two weeks. The

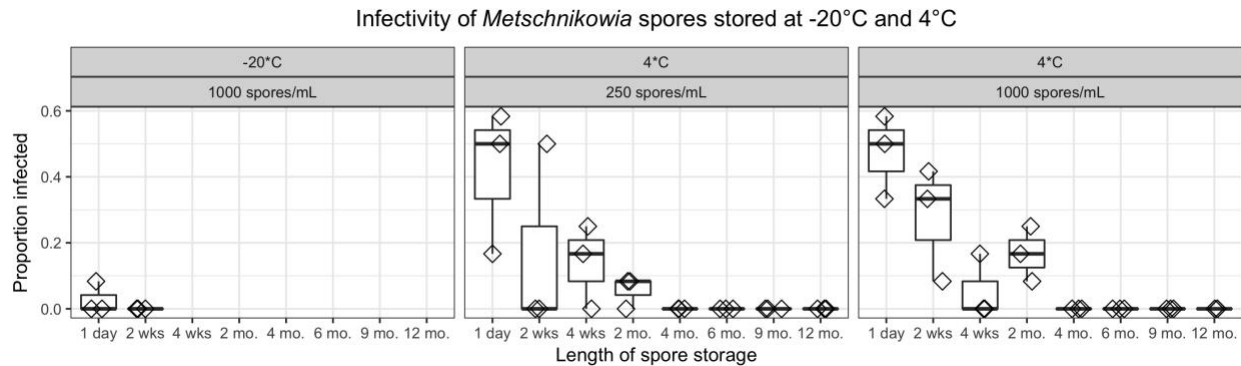
214 infectivity of *Metschnikowia* spores that had been stored at 4°C declined over the first two

215 months of storage and, by four months, spores were no longer infective. In our analysis of the

216 *Metschnikowia* 4°C treatment data, only storage length significantly impacted infectivity (dose:  $z$

217 = 0.280,  $p = 0.780$ ; storage length:  $z = -3.58$ ,  $p = 0.0003$ ; interaction:  $z = 0.840$ ,  $p = 0.401$ ).

218



219

220 **Figure 3.** Spores of the fungus *Metschnikowia bicuspidata* rapidly lost infectivity.

221 *Metschnikowia* spores were killed within days at -20°C (left panel) and infectivity declined

222 sharply over 2 months at 4°C (center and right panels).

223

224 At 4°C, spore yield from *Metschnikowia*-infected hosts did not change significantly with

225 storage length ( $t = -1.25, p = 0.229$ ; Figure 2). However, the trend was similar to that in

226 *Pasteuria*-infected hosts (Figure 2), and the lack of significance may simply reflect lower power

227 based on having only three replicate tubes for *Metschnikowia* vs. five for *Pasteuria* (Table 1).

228

## 229 Discussion

230 Infectivity and spore yield of two commonly studied *Daphnia* parasites were strongly

231 influenced by storage length and temperature. Spores of the bacterium *Pasteuria ramosa*

232 survived and remained infective for at least a year in both the refrigerator and the freezer.

233 However, spore yield from *Pasteuria*-infected hosts unexpectedly declined over time, especially

234 in the refrigerator. Spores of the fungus *Metschnikowia bicuspidata*, on the other hand, were

235 rapidly killed by freezing; when stored in the refrigerator, *Metschnikowia* infectivity declined

236 sharply over the first two months of storage and, by four months, spores were no longer

237 infective. Spore yield of *Metschnikowia*-infected hosts trended downwards, but was not  
238 significantly impacted by storage length. Thus, when using these parasites in lab experiments,  
239 protocols should take into account the effects of storage length and temperature on spore  
240 infectivity and yield.

241         The differential response of *Pasteuria* and *Metschnikowia* spore infectivity to storage has  
242 important implications for experimental design (Figures 1 and 3). While *Pasteuria* spores can be  
243 stored in the lab for relatively long periods (months to years) before being used to infect hosts,  
244 *Metschnikowia* spores lose their infectivity over a period of weeks to months. Because of this,  
245 our lab always seeks to use *Metschnikowia* spores that are less than one month old for  
246 experiments. This presents challenges for experiments exposing field-collected hosts to field-  
247 collected spores. Such experiments require growing enough individuals of each host genotype in  
248 the lab prior to spore exposure. Ideally, these hosts would be grown for several generations under  
249 standardized conditions to control for maternal effects (i.e., influences of the mother's  
250 environment on an offspring's phenotype). However, experiments with field-collected hosts and  
251 spores must trade leaving more time to grow more host individuals against the rapid loss of  
252 infectivity of the field-collected spores.

253         The rapid death of *Metschnikowia* in the lab raises questions about how spores survive in  
254 the environment between epidemic outbreaks. *Metschnikowia* dynamics in lake populations are  
255 strongly seasonal, with disease outbreaks occurring in late summer and autumn (Duffy et al.  
256 2009). Moreover, in the *Daphnia*–*Metschnikowia* system that has been best studied, the host,  
257 *Daphnia dentifera*, is dormant in the sediment for several months of the year. Thus,  
258 *Metschnikowia* is presumably able to remain infective in sediments longer than in  
259 microcentrifuge tubes in the lab. Indeed, it is possible to take sediment from a lake that had an

260 epidemic in a previous year and use it to start epidemics in whole-water column enclosures in a  
261 lake that did not have an epidemic (A.J. Tessier, personal communication).

262 *Metschnikowia*'s intolerance of freezing might help explain patterns of disease in natural  
263 populations. While *Metschnikowia* has been found in ponds (Green 1974, Codreanu &  
264 Codreanu-Balcescu 1981), its prevalence in those habitats tends to be low (<4%) (Stirnadel &  
265 Ebert 1997, Goren & Ben-Ami 2013). In stratified lakes, on the other hand, *Metschnikowia*  
266 prevalence can be quite high, reaching 20% – 40% (Hall et al. 2011, Strauss et al. 2016).  
267 *Metschnikowia*'s intolerance of freezing might be a factor driving this pattern, as the sediments  
268 in ponds are much more likely to freeze. However, it is likely that other factors—such as  
269 different predation environments and stratification itself—also influence the relative prevalence  
270 in lakes vs. ponds (Auld et al. 2014a).

271 Spore yield from infected hosts is an important component of parasite fitness and, as a  
272 result, is frequently measured in studies of these two parasites (e.g., Jensen et al. 2006, Duffy et  
273 al. 2011, Auld et al. 2014a, Auld et al. 2014b). Our results suggest that the spore yield from  
274 infected hosts decreases over time, especially if the hosts are stored in the refrigerator (Figure 2).  
275 Ideally, spore counts from infected hosts should be done as soon after collection as possible.  
276 However, if spore counts cannot be done quickly (within days to weeks), our results suggest that  
277 samples should be stored in the freezer until they can be counted to reduce spore loss (for  
278 *Metschnikowia*, this should only be done if the spores are not needed for future infections). Even  
279 with freezer storage, spore yield will decline over time, so it is especially important to intersperse  
280 individuals from different treatments when counting samples, rather than counting all individuals  
281 from a single treatment before moving on to the next.

282 Our experiment used one isolate of *Pasteuria* and one isolate of *Metschnikowia*. It is  
283 possible that genotypes differ in their ability to tolerate storage under lab (and field) conditions.  
284 We hypothesize that such variation is more likely in *Pasteuria* than in *Metschnikowia*, since  
285 *Pasteuria* shows substantial variation (Carius et al. 2001, Mouton & Ebert 2008, Auld et al.  
286 2012), whereas *Metschnikowia* shows strikingly little phenotypic and genotypic variation (Duffy  
287 & Sivars–Becker 2007, Wolinska et al. 2009, Searle et al. 2015). However, whether either  
288 parasite contains genetic variation related to tolerance of storage conditions remains to be tested.

289 *Daphnia* and their microparasites have emerged as an important study system for  
290 understanding the ecology and evolution of infectious diseases (Ebert 2005, Lampert 2011,  
291 Cáceres et al. 2014). Our study provides valuable information on how storage length and  
292 temperature influence spore infectivity and yield from infected hosts. When designing  
293 experiments, scientists should take into account that spore yield of *Pasteuria* declines over time,  
294 especially at 4°C, and that *Metschnikowia* spores are killed rapidly at -20°C and die within  
295 weeks to months at 4°C.

296

## 297 **Acknowledgments**

298 This work was supported by the US National Science Foundation (DEB-1353806). We thank  
299 Carla Cáceres for helpful discussions and Sarah Boon for comments on this manuscript.

300

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