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► **To cite this version:**

Apostolos-manuel Koussoroplis, Erik Sperfeld, Sylvain Pincebourde, Alexandre Bec, Alexander Wacker. Dietary sterol availability modulates heat tolerance of *Daphnia*. *Freshwater Biology*, in-Press, 10.1111/fwb.14037 . hal-03969167

HAL Id: hal-03969167

<https://hal.science/hal-03969167>

Submitted on 2 Feb 2023

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1 Dietary sterol availability modulates
2 *Daphnia*'s heat tolerance

3

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15

16 **Keywords:** climate change, cyanobacteria, cholesterol, lakes, nutritional ecology, thermal
17 stress, zooplankton.

18

19

20 **Abstract** (max 350 words)

- 21 1. The increasing frequency and intensity of summer heat waves is pushing freshwater
22 zooplankton towards its upper thermal tolerance limits. At the same time, higher
23 temperatures and prolonged water column stratification can favor the dominance of
24 cyanobacteria in phytoplankton. Even when not toxic or grazing resistant, these
25 prokaryotes lack phytosterols as essential precursors for cholesterol, the main sterol in
26 animal tissues. Cholesterol plays a crucial role in the physiological adaptation of
27 ectotherms to high temperature. Therefore, the shift to cyanobacteria-dominated
28 systems may increase the vulnerability of zooplankton to heatwaves by intensifying
29 cholesterol limitation.
- 30 2. Here, we use death time curves that take into consideration the intensity and duration
31 of a thermal challenge and a dynamic model to study the effects of cholesterol
32 limitation on the heat tolerance of the keystone species *Daphnia magna* and to
33 simulate the cumulative mortality that could occur in a fluctuating environment over
34 several days of heatwave.
- 35 3. We show that increasing cholesterol limitation does not affect the slope between time-
36 to-immobilization and temperature, but does decrease the maximal temperature that
37 *Daphnia* can withstand by up to 0.74°C. This seemingly small difference is sufficient
38 to halve the time individuals can survive heat stress.
- 39 4. Our simulations predict that, when facing heatwaves over several days, the differences
40 in survival caused by cholesterol limitation build up rapidly. Considering the
41 anticipated intensity and duration of future (2070-2099) heatwaves, cholesterol
42 limitation could increase mortality by up to 45% and 72% under low- and medium-
43 greenhouse-gas-emission scenarios, respectively.
- 44 5. These results suggest that the increasing risk of cholesterol limitation due to more
45 frequent cyanobacterial blooms could compromise the resistance of zooplankton

46 populations to future heatwaves. More generally, this study underscores the
47 importance of considering the nutritional context in any attempt to predict ectotherm
48 mortality with increasing temperatures in the field.

49 **Introduction**

50 “Summertime is quickly becoming a deadly season on earth” (Stillman, 2019). As
51 mean temperature and variance rise under the effect of global warming (Diffenbaugh and
52 Field, 2013; Donat and Alexander, 2012; Easterling et al., 2000) summertime heatwaves
53 become increasingly frequent, intense and long-lasting. During these extreme events,
54 temperatures can rise beyond the physiological limits of ectotherms causing stress, mortality
55 and under some circumstances, alterations of populations and ecosystems (Bailey and van de
56 Pol, 2016). Thermal tolerance, i.e. the ability of an organism to withstand extreme
57 temperatures is thus an important trait for forecasting species’ future persistence and
58 distribution (Gunderson and Stillman, 2015; Kelly et al., 2012; Sunday et al., 2014).

59 While thermal tolerance is phylogenetically constrained (Hoffmann et al. 2013), it can
60 also be adjusted to some extent within the life-time of an individual (Hoffmann et al., 2013;
61 Yampolsky et al., 2013). Such plasticity is achieved through a series of physiological
62 mechanisms including (1) the reduction of protein denaturation via up-regulation of heat
63 shock proteins (HSP, Richter et al., 2010), (2) the reduction of oxidative stress at high
64 temperatures *via* the up-regulation of antioxidant pathways (Lushchak, 2011), and (3) the
65 maintenance of cell membrane structural properties at high temperatures via membrane lipid
66 remodeling (Hazel, 1995; Sinensky, 1974). When it comes to high temperatures, cholesterol
67 plays a major role in membrane functioning. Cholesterol is an important structural component
68 of cell membranes in animals. It stabilizes membrane structure (Bloom and Mouritsen, 1988),
69 limits membrane permeability (Kroes and Ostwald, 1971), and imparts a suitable working
70 environment for membrane proteins (Yeagle et al., 1988). In wild copepod populations, the
71 cholesterol content is positively correlated with maximum habitat temperature (Hassett and

72 Crockett, 2009). As for most arthropods (Behmer and Nes, 2003), cholesterol is an essential
73 nutrient for crustacean zooplankton (von Elert et al., 2003). This means that it cannot be
74 synthesized *de novo* and that cholesterol, or its sterol precursors (e.g. phytosterols) have to be
75 supplied by food intake (Martin-Creuzburg and von Elert, 2004). Experimental studies on the
76 microcrustacean *Daphnia* have demonstrated that dietary cholesterol limitation impairs
77 somatic growth at higher temperatures (Martin-Creuzburg et al., 2012; Sperfeld and Wacker,
78 2009). Cholesterol supply is also suspected to influence heat tolerance (i.e. the critical thermal
79 limits) (Hassett and Crockett, 2009), but this remains to be tested experimentally.

80 In the planktonic environment, sterols are only synthesized by eukaryotic
81 microorganisms (auto- and heterotrophic protists), while prokaryotes such as cyanobacteria
82 are completely devoid of these compounds. Even among eukaryotes however, many common
83 green algae *genera* contain either low sterol concentrations or sterols that cannot be converted
84 into cholesterol (Martin-Creuzburg et al., 2014; Martin-Creuzburg and Merkel, 2016). Hence,
85 green algae and most importantly, cyanobacterial dominance in lake phytoplankton can
86 drastically increase the risk of sterol limitation for zooplankton, in particular for non-selective
87 filter feeders such as most cladocerans which cannot preferentially feed on sterol-rich species.

88 Here we hypothesize that dietary sterol limitation reduces heat tolerance in a key
89 freshwater zooplankton species, the model organism *Daphnia magna*. In that aim, we
90 experimentally generated thermal death time (TDT) curves of a *D. magna* clone supplied
91 with different amounts of cholesterol. Unlike most analyses on heat tolerance that implicitly
92 assume that critical temperatures remain constant through time, the TDT framework
93 (Jørgensen et al., 2021, 2019; Peralta-Maraver and Rezende, 2020; Rezende et al., 2014) takes
94 into consideration the intensity and duration of a thermal challenge. Applying this framework,
95 we were able to test whether and how cholesterol modulates the dependence of thermal death
96 time on exposure temperature and duration. The major advantage of TDT curves over

97 previously used assessments of heat tolerance at a single temperature is that it enables the
98 prediction of survival in a more realistic, variable environment, such as diurnal fluctuations
99 experienced by ectotherms (Rezende et al., 2020a). Hence, in a second step, we used a
100 recently developed model (Rezende et al., 2020a) and heat wave projections for lakes
101 (Woolway et al., 2021) to predict how changing cholesterol availability can modulate *D.*
102 *magna* mortality under heat waves of various intensity and duration. We caution that these
103 simulations cannot be used as accurate prediction of *D. magna* mortality rates in the field
104 under future scenarios of heat waves. Instead we seek to convey the message that even small
105 differences on heat tolerance induced by cholesterol supply are sufficient to cause large
106 differences in survival as heatwaves last increasingly longer and are more intense with climate
107 change.

108 **Methods**

109 *Theoretical framework*

110 A detailed description of the TDT framework can be found in (Rezende et al., 2014, 2020a).
111 Briefly, the framework is based on the premise that the accumulation of heat-induced injury
112 varies with extreme temperature and exposure duration. A TDT describes the time until an
113 organism's immobilization (t_{imm}) as a function of experimental temperature (T) following:

$$114 \log_{10} t_{imm} = \frac{CT_{max}}{z} - \frac{1}{z} T \quad \text{eq (1)}$$

115 Which can also be rearranged as:

$$116 T = -z \log_{10} t_{imm} + CT_{max} \quad \text{eq (2)}$$

117 Where CT_{max} corresponds to the maximum critical temperature (i.e., temperature at which
118 coherent motion is lost) for an exposure of one minute and z estimates the sensitivity of t_{imm} to
119 temperature change. For example, for an organism with $z = 1^\circ\text{C}$, an increase of 1°C in
120 temperature will lead to a 10-fold decrease in t_{imm} . Regarding heat tolerance, species with a
121 higher CT_{max} can – at least for a short period of time – tolerate higher temperatures, whereas

122 lower z values indicate that even large temperature changes have little impact on t_{imm} . Most
 123 importantly however, eq. 2 indicates how to estimate CT_{max} and z by fitting a linear model to
 124 t_{imm} -by-experimental T data (**Fig. 1a**).

125 As in most cases, in our study (*see* methods below), t_{imm} is the arithmetic average of a
 126 static assay at a given experimental temperature so that the TDT line modelled by **eq. 2**
 127 represents the 50% survival isocline of a response surface of survival probability as a function
 128 of experimental temperature and exposure time (*i.e.* the **tolerance landscape** *sensu* (Rezende
 129 et al., 2014)). Assuming that the survival probability at any temperature is described by a
 130 single function $S(t)$, from eq. 2 it follows that the difference in the time needed to reach the
 131 same value of relative survival (e.g. 50%) between two temperatures can be expressed as
 132 follows:

$$133 \quad \frac{t_{imm_2}}{t_{imm_1}} = 10^{\frac{-(T_2-T_1)}{z}} \quad \text{eq. (3)}$$

134 Eq. 3 implies that any survival probability function $S(t)$ shifts horizontally by $10^{\frac{1}{z}}$ for every
 135 °C of temperature increase. Based on this premise, (Rezende et al., 2020a) developed an
 136 algorithm that uses static assay data to predict cumulative survival over a period of thermal
 137 fluctuation, such as a natural diurnal temperature cycle. The algorithm first constructs a
 138 thermal tolerance landscape with $S(t)$ as response variable and assay time and temperature as
 139 predictor variables. The algorithm does not assume a particular mathematical function for
 140 $S(t)$, but fits instead a survival curve to the survival data obtained at each assay temperature.
 141 The curves from each assay temperature T , $S(t)_T$, are then collapsed at a single reference
 142 temperature, T_{ref} , using eq. 3 and averaged to produce $\overline{S(t)_{T_{ref}}}$. The tolerance landscape,
 143 $S(t, T)$, is then constructed using eq. 3 again such as:

$$144 \quad S(t, T) = \overline{S(t)_{T_{ref}}} 10^{\frac{-(T_{ref}-T)}{z}} t \quad \text{eq. (4)}$$

145 We applied this theoretical framework to (1) assess the effects of cholesterol supply on the
146 heat tolerance of *Daphnia* under static conditions, and (2) determine the consequence of the
147 effect of cholesterol limitation on thermal tolerance for the probability to survive climate
148 change-induced heat waves.

149 *Organism culture and nutritional conditioning*

150 Stock cultures of *D. magna* were kept as in (Koussoroplis and Wacker, 2016). For the
151 experiments we used *Synechococcus elongatus* (SAG 89.79), a well edible, non-toxic
152 cyanobacterium lacking essential sterols and polyunsaturated fatty acids as base food source.
153 *S. elongatus* was offered in food concentration of 2 mg carbon (C) per liter, which is well
154 above the incipient threshold for food limitation in *Daphnia magna* (~0.5 mg C l⁻¹ (Lampert
155 and Muck, 1985)), and then supplemented with different amounts of cholesterol-containing
156 liposomes to obtain four cholesterol concentrations of 0.25, 2.5, 5, and 8 µg per mg C. In all
157 treatments, a small amount of eicosapentaenoic acid (20:5ω3) was also added via liposomes
158 (resulting in 0.25 µg 20:5ω3 per mg C) in order to prevent severe 20:5ω3 limitation, thereby
159 aiding the development of daphnids. All liposomes were produced following the protocol
160 described in (Wacker and Martin-Creuzburg, 2012).

161 For the experiment, we used third-clutch juveniles of *D. magna* (< 12 h old) from
162 mothers kept at 20°C and fed on *Tetradesmus obliquus* (SAG 276-3a), a green alga containing
163 sterols. The neonates were born on pure *S. elongatus* at 20°C and subsequently randomly
164 allocated to jars containing the different dietary treatments at a density of 30 ind. l⁻¹.
165 Afterwards temperature was elevated progressively to 27°C within 6 h, and the daphnids were
166 kept at 27°C for seven days when the thermal tolerance assays took place. The rationale
167 behind this temperature choice is based on the fact that heat tolerance in *Daphnia* is positively
168 correlated to acclimation temperature (Burton et al., 2018) and that in nature, *Daphnia*
169 populations experiencing heat stress above 30°C are more likely to have experienced high

170 average temperatures during preceding days. An average water temperature of 27°C is
171 ecologically relevant for small north temperate lakes and ponds during summer months
172 (Andersen et al., 2017; Woolway et al., 2021). During the acclimation period, the daphnids
173 were transferred daily to new jars with freshly prepared food suspensions.

174 *Thermal tolerance assays*

175 At day seven, *Daphnia* from the different jars were pooled within food treatments in
176 order to remove a potential jar effect and afterwards randomly chosen and individually placed
177 in 1.5 ml Eppendorf tubes without food and randomly assigned to heat blocks set at the
178 different constant temperatures (34.5°, 35.5°, 36.5°, 37.5°, 38.5°C). Preliminary experiments
179 showed that the chosen temperatures were satisfying the logistic constraints of an assay
180 lasting less than 8h in order to minimize the risk of starvation or hypoxia effects on mortality
181 and more than 10 min, which was the minimal time that was required for controlling all assay
182 tubes. For the highest quality diet (8 µg chol. mg C⁻¹), we could test 8-10 individuals at each
183 temperature, whereas for all other diets we tested 4-5 individuals per temperature due to
184 mortality losses. During the assay, all individuals were continuously monitored visually.
185 Thermal immobilization was concluded when the individuals did not move for more than 10
186 sec and did not respond to gentle physical stimuli, i.e. by gentle prodding the tube. Once
187 immobilized, the t_{imm} was recorded and the organism's length (from the top of the head to the
188 basis of the tail spine) was measured (to the nearest µm using a stereomicroscope) as a proxy
189 for body size.

190 *Data analysis*

191 In order to assess the effects of cholesterol supply on the heat tolerance under static
192 conditions, we tested whether the linear model below including cholesterol supply effects on
193 both intercept and the slope of the TDT provides a better fit than models without these effects:

$$194 \quad T = \beta_0 + \beta_t \log_{10} t_{imm} + \beta_C Chol + \beta_{tC} \log_{10} t_{imm} Chol \quad \text{eq. (5)}$$

195 Where β_0 , β_t , β_C , β_{tC} are the model coefficients associated with the intercept, the t_{imm}
196 ,cholesterol supply (as a factor) and their interaction, respectively. We employed the second-
197 order Akaike Information Criterion (AIC_c) to select the model that best fit the data. Given eq.
198 2 and the full model in eq. 5, it follows that $CT_{max}(Chol) = \beta_0 + \beta_C Chol$ and that
199 $-z(Chol) = \beta_t + \beta_{tC} Chol$. Diagnostics for heteroscedasticity and normality were inspected
200 from residual plots produced from each of the three fitted models. The significance of the
201 effects of cholesterol supply on thermal tolerance was tested by type II ANOVA in order to
202 account for the unequal sample sizes between the 8 μg chol. $mg\ C^{-1}$ cholesterol treatments and
203 the others (Langsrud, 2002).

204 Despite our efforts to distribute the daphnids from each cholesterol treatment
205 randomly among temperature treatments, individuals assayed at 34.5°C and 38.5°C tended to
206 be smaller than at the other temperatures (Supplementary material, Fig. S2). Size, and more
207 particularly body mass, can influence thermal tolerance (Peralta-Maraver and Rezende, 2020).
208 By regressing size versus t_{imm} independently for each assay temperature – cholesterol
209 treatment combination we found no size-dependency in t_{imm} in almost all of the 20
210 treatments, except the 34.5°C - 0.25 μg Chol $mg\ C^{-1}$ and 34.5°C - 8 μg Chol $mg\ C^{-1}$, for
211 which marginally significant positive correlations were found (Supplementary material, Fig.
212 S3). Yet, (Burton and Einum, 2020) found that in *Daphnia magna*, size had a significant
213 effect on CT_{max} . In order to verify that size did not alter our conclusions on the effect of the
214 cholesterol supply we also tested the inclusion of size as a second continuous variable in eq. 5
215 (see Supplementary Material).

216 In order to assess the effects of cholesterol supply on the heat tolerance under
217 fluctuating conditions (see below), our dataset was subsequently used to model a thermal
218 tolerance landscape (*see eq. 5*) for each food quality using the *tolerance.landscape* function of
219 the R scripts made available in DRYAD by (Rezende et al., 2020b). The theoretical estimates

220 from the tolerance landscapes were regressed against the observed data in order to evaluate
221 the quality of the models.

222 *Predicting survival under climate change-induced heat waves*

223 We explored the consequence of the effect of cholesterol limitation on thermal
224 tolerance for the probability to survive climate change-induced heat waves. Our aim here is to
225 illustrate the extent to which cholesterol limitation may matter for survival during heat wave
226 events in the future – we highlight that these predictions cannot be validated since the field
227 data allowing this validation does not exist yet. We generated four temperature time series
228 simulating heatwaves varying in duration and intensity. The time series simulated the average
229 duration and intensity observed in an average northern temperate (latitude: 23.5° N-66.5° N)
230 shallow lake (< 5m) under historical (1901-2005) and future (2070-2099) climate forcing
231 based on three greenhouse-gas-emission scenarios (Representative Concentration Pathway
232 (RCP), RCP 2.6, RCP 6.0, and RCP 8.5). To create the time series, we extracted the average
233 lake surface temperature climatology for northern temperate lakes reported in (Woolway et
234 al., 2021 - Extended data Fig. 2e). The heatwave average intensity (i.e. °C above base line
235 climate) and duration for shallow lakes was also extracted from (Woolway et al., 2021 -
236 Extended data Fig. 4c). The extracted average intensities (Fig. 3a) were then numerically
237 added to the climatology data for the corresponding extracted average durations (Fig. 3a),
238 thereby creating time series of average daily temperature. Finally, we incorporated the daily
239 temperature fluctuation signal around these average temperatures by assuming an 8°C daily
240 cycling range (*i.e.* mean daily average $\pm 4^\circ$ C; Woolway et al., 2016). The simulated time
241 series were all centered at day 235, which is roughly the middle of northern hemisphere
242 summer (Fig. 3b) and were used to predict *Daphnia* heatwave tolerance. The survival during
243 the simulated heatwaves was predicted using the *dynamic.landscape* function (Rezende et al.,
244 2020b), which takes as arguments the *tolerance.landscape* function output and a temperature

245 time series. The survival rate was predicted day-by-day. Assuming a complete recovery of
246 surviving individuals during nighttime, the cumulative survival rate over the total duration of
247 the heatwave was calculated as the product of daily survival rates. The uncertainty around the
248 predictions was estimated by non-parametric bootstrapping (Efron and Tibshirani, 1993) of
249 the experimental datasets used to produce the *tolerance.landscape* function outputs (n=100).
250 All data analyses and simulations were performed using R (R Development Core Team,
251 2008).

252 **Results**

253 Daphnids from the four dietary cholesterol treatments did not differ in length before
254 the start of the thermal tolerance assays (Supplementary material, Fig. S1).

255 The model with the best fit according to the AIC_c was the one including both t_{imm} and
256 *Chol* but not their interaction (table 1, adjusted $r^2=0.873$, $p < 0.001$). This result implies that
257 cholesterol supply significantly impacts CT_{max} (Table 2), but not z , which remains constant
258 across food treatments. The value of z was estimated at 2.35°C [95% CI: $2.6-2.02^\circ\text{C}$] and
259 CT_{max} varied from 40.14°C [95% CI: $39.83-40.46^\circ\text{C}$] for the lowest cholesterol supply to
260 40.85°C [95% CI: $40.29-41.41^\circ\text{C}$] for the highest cholesterol level (Fig. 1b, Table 3).
261 Including size as predictor variable in the model indicated that it had a significant effect on
262 heat tolerance but this did not remove the significant effect of cholesterol (Table S1). The
263 CT_{max} for average- sized individuals in the different cholesterol treatments were slightly
264 offset ($+0.5^\circ\text{C}$) but the differences between the cholesterol treatments remained similar (Fig.
265 S4).

266 We tested the suitability of the algorithm provided by Rezende et al. (2020a) to predict
267 the thermal tolerance landscape across the cholesterol supply levels. Overall, the estimates
268 from the modelled tolerance landscapes resembled the observed data ($R^2 = 0.87-0.98$; Fig. 2),
269 but the quality of the fit depended on cholesterol supply. The best fits were found for the

270 lowest cholesterol supply treatments (0.25 and 2.5 $\mu\text{g chol. mg C}^{-1}$). For the higher cholesterol
271 treatments, the modelled data tended to underestimate t_{imm} at 37.5°C while it overestimated
272 it at 38.5°C (Fig. 2).

273 Under the different heat wave scenarios (Fig. 3A-B), the models predicted between
274 >0.9 [95% CI: 0.902-0.916] (historical scenario) and 0.14 [95% CI: 0.127-0.151] (RCP 8.5)
275 cumulative survival for the highest cholesterol supply (Fig. 3C). The models predicted that
276 cholesterol supply had a marked effect on heat wave survival. Under the historical scenario,
277 survival dropped to 0.76 [95% CI: 0.748-0.768] for the lowest cholesterol supply. Under the
278 RCP 2.6 (or 6.0) scenario, survival dropped from 0.72 [95% CI: 0.704-0.731] (0.54 [95% CI:
279 0.522-0.556]) to 0.38 [95% CI: 0.367-0.499] (0.17 [95% CI: 0.158-0.188]) between the
280 highest and the lowest cholesterol supply level. Under the most pessimistic RCP 8.5 scenario,
281 the model predicted very low survival at the higher cholesterol supply treatments (<0.20) and
282 a complete extinction at low cholesterol supply (Fig. 3C).

283 Discussion

284 By the end of the 21st century the duration and intensity of heat waves in lakes is
285 expected to increase dramatically (Woolway et al., 2021). Our results indicate that cholesterol
286 supply likely modulate the impact of such heat waves on *Daphnia* populations by altering
287 individual thermal tolerance. To date, the link between cholesterol supply and heat tolerance
288 was hypothetical and based on correlations between habitat temperature, body sterol content
289 and heat tolerance across various copepod species (Hassett and Crockett, 2009). While we did
290 not find an effect of cholesterol supply on the temperature sensitivity parameter z , we show
291 that cholesterol limitation can decrease the maximal temperature that *Daphnia* can withstand
292 (CT_{max}) by up to 0.74°C. This apparently minute difference halves the average t_{imm} (see x-
293 axis in **Fig. 1B**). For example, at 34°C, a temperature that is not unlikely in surface waters of

294 small ponds (Andersen et al., 2017b; Vilas et al., 2018), the t_{imm} of our clone drops from
295 13.7 to 6.8 hours, an effect that can substantially influence survival during heatwaves.

296 The effects of cholesterol on the functioning of cell membranes are manifold: it
297 contributes to maintenance of membrane order and of the liquid crystalline phase, but also
298 interacts directly with membrane proteins such as Na⁺, K⁺, -ATPase thereby modulating their
299 activity (Crockett, 1998). All these processes are affected by temperature and are
300 compromised during heat stress. When reared at higher temperatures, *Daphnia* tend to
301 increase the cholesterol content in their tissues indicating a higher demand (Sperfeld and
302 Wacker, 2009). However, body cholesterol homeostasis in *Daphnia* is weak and strongly
303 depends on cholesterol supply, i.e. even if somatic growth rate becomes limited, the
304 cholesterol content of *Daphnia* keeps decreasing with decreasing supply (Sperfeld and
305 Wacker, 2009). Reduced growth did not preserve tissue cholesterol content, as one would
306 expect, suggesting that heat tolerance (as mediated by membrane cholesterol content) could
307 be traded-off for somatic growth under cholesterol limitation.

308 An unexpected result was the absence of a cholesterol effect on somatic growth (i.e.
309 no size differences at day seven). This contrasts with previous studies where cholesterol
310 limitation decreased *Daphnia* growth, particularly at higher temperatures (Sperfeld and
311 Wacker, 2009). One possible explanation for that is that despite our precautions to ensure
312 sufficient food during the nutritional conditioning phase, the combination between relatively
313 high *Daphnia* density and the high temperatures might have led to a limitation by food
314 quantity towards the end. Indeed, it is possible that daphnids in the highest cholesterol
315 treatments initially grew faster and reached earlier a size enabling them to deplete their food
316 faster than it was renewed. This would have allowed the daphnids from other treatments to
317 catch-up in size. Under this assumption, it is possible that the decrease of 0.74°C is at the
318 lower range of the effect size of cholesterol supply on CT_{max} . Yet, here again, this apparently

319 minute decrease has consequences. It yields a 16% decrease in cumulative survival in our
320 “historical” scenario simulation. The consequences become dramatic when considering future
321 heatwaves where cumulative survival decreases by 45% in RCP2.6 and 72% in RCP6.0
322 scenarios. In the worst-case scenario (RCP8.5), this 0.74°C decrease in CT_{max} , leads to a
323 complete extinction. This is explained by the fact that cumulative mortality grows
324 exponentially with time and small differences in daily mortality quickly build-up as time
325 passes by as shown in *Drosophila* (Rezende et al. 2020a).

326 These simulations underscore the importance of considering the nutritional context in
327 any attempt to predict ectotherm temperature mortality in the field. In the case of *Daphnia*,
328 studies on heat tolerance often use commercial shellfish diets based on high quality marine
329 microalgal species (Burton et al., 2020, 2018; Burton and Einum, 2020). These sterol-rich
330 diets greatly contrast with what will be available to *Daphnia* under future climates. In lakes,
331 the availability of cholesterol or suitable phytosterol precursors for filter feeding zooplankton
332 such as *Daphnia* is controlled by the taxonomic composition of phytoplankton communities
333 (Martin-Creuzburg and Merkel, 2016). Cyanobacteria and chlorophytes share similar and high
334 temperature optima for growth (around 29°C) (Carey et al., 2012; Lüring et al., 2013).
335 Hence, the increase in average lake temperatures is expected to promote chlorophytes and
336 cyanobacterial blooms through direct temperature effects of and increased water column
337 stability (which favors cyanobacteria) (Jöhnk et al., 2008; Kosten et al., 2012; Smucker et al.,
338 2021). Cyanobacteria completely lack sterols and certain common species of chlorophytes do
339 not contain sufficient concentrations in the appropriate phytosterol pre-cursors of cholesterol
340 (Martin-Creuzburg and von Elert, 2009). Furthermore, cyanobacterial blooms and longer
341 durations of stratification create additional problems by causing hypoxia in deeper waters
342 (Andersen et al., 2017a; Smucker et al., 2021). Thus, the cooler water at depth might not be
343 available as a thermal refuge for aquatic animals (Till et al., 2019), especially if feeding on

344 low quality diets that can increase their oxygen demand (Ruiz et al., 2021), thereby leaving no
345 other choice but the deadly hot surface waters (Vad et al., 2013). Hence, considering the
346 positive correlation between heatwave severity (intensity and duration) and the risk of
347 cholesterol limitation via cyanobacterial blooms yields much more pessimistic temperature-
348 driven mortality projections for *Daphnia* populations in the field.

349 Our results are rough estimates and need to be considered with caution. First, our
350 simulations are limited to northern temperate shallow (< 5m) lakes, close to the small fishless
351 ponds in which *D. magna* is typically found. The increase in heatwave intensity is expected to
352 be somewhat lower for lower latitudes and deeper lakes (Woolway et al., 2021). The
353 heatwave duration should however show the opposite patterns, with longer lasting heatwaves
354 at low latitudes and in deep lakes (Woolway et al., 2021). As we discussed above, small
355 differences in daily mortality grow exponentially with time. It is therefore possible that the
356 longer heatwave duration in deeper lakes will compensate for the lower heatwave intensity,
357 yielding similar or even worse cumulative mortalities. Second, the model we used to predict
358 cumulative mortality has limitations that need to be considered. In its current form, it assumes
359 that surviving individuals recover completely from heat injury during the cool phase
360 overnight. Yet, it has been shown that repeated exposure to near-lethal temperatures can
361 progressively lower thermal limits (Siegle et al., 2018). Under such circumstances, our model
362 likely underestimates cumulative mortality (Jørgensen et al., 2021, 2019) and predictions are
363 therefore more conservative. Further experiments are therefore necessary in order to gain a
364 better understanding of thermal injury and recovery dynamics in daphnia and ectotherms in
365 general. In addition, our purpose was to quantify the interaction between cholesterol supply
366 and temperature, but thermal tolerance can also be modulated by various abiotic (dissolved
367 oxygen, pH, etc.) and biotic (pathogens, macronutrients, other limiting micronutrients such as
368 amino acids) factors. Heat tolerance can also vary between clones (Sarrazin and Sperfeld,

369 2022) and a natural population rarely consists of a single clone. Hence, in nature the response
370 of the population might be different than reported here. A final word of caution is also
371 required on the fact that mortality alone cannot be used to extrapolate population trends
372 without considering the impact of heatwaves on reproduction. Temperature and diet are
373 known to interactively influence reproductive traits in ectotherms (Kim et al., 2020; Ruiz et
374 al., 2020; Sperfeld and Wacker, 2012) and heat stress will likely also negatively affect
375 reproductive output. At high cholesterol concentrations however, *Daphnia* may reproduce
376 enough offspring to sustain positive population growth no matter the (mortality) scenario. On
377 the other hand, reproduction is lower under low cholesterol supply, likely exacerbating the
378 effects of heat waves on population growth (Sperfeld and Wacker, 2009).

379 Heat wave events are increasingly challenging natural systems, including the
380 ecophysiology of ectotherms and their ability to tolerate high temperatures (Ma et al., 2021).
381 The thermal inertia of lakes and aquatic systems in general will not buffer the large amplitude
382 of warming during heat wave events (Woolway et al., 2021). Our results suggest that adequate
383 cholesterol supply may help daphnids to tolerate heat wave conditions in the intermediate
384 greenhouse gas release scenarios for climate change. This beneficial effect will likely
385 disappear in the worst climate change scenario. Large mortality events in a keystone
386 zooplankton organism can have dramatic consequences on the functioning of freshwater
387 ecosystems and the associated services they provide. Nutritional limitations should therefore
388 be incorporated into predictive models of climate change impacts.

389 **Acknowledgements:**

390 The authors are grateful to Silvia Heim for technical assistance. This study was supported by the
391 German Research Foundation (DFG, KO5330/1-1 to AMK).

392 **Conflict of interest:**

393 None

394 **Author contributions:**

395 Conceptualisation, developing methods, and conducting the research: AMK and AW. Data
396 analysis: AMK with substantial help from all authors. Figure preparation: AMK. Writing:
397 AMK, ES, AW, AB, SP

398 **Data availability statement**

399 Should the paper be accepted for publication, data and R-scripts will be made available at the
400 Dryad Digital Repository

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573 **Tables**

574 **Table 1:** Set of candidate linear models testing the relationship between critical body
 575 temperature (T_{crit}) in *Daphnia magna* as a function of exposure time (t) and cholesterol
 576 supply during dietary preconditioning. df : degrees of freedom; ΔAIC_c : difference in AIC
 577 between a given model and the best fitting model of those considered; w_i : probability that a
 578 given model is the best among those considered. is the best among those considered.

Model	Predictor	df	$logLik$	AIC_c	ΔAIC_c	w_i	R^2
1	Time	3	-100.554	207.3	19.91	0.000	0.844
2	Time+Cholesterol	6	-87.969	187.4	0	0.723	0.874
3	Time*Cholesterol	9	-84.969	189.4	2.02	0.267	0.874

579

580 **Table 2:** type II ANOVA results and parameter estimates for the selected model (see table 1)

	df	SS	F	p
Time	1	234.41	906.10	<2.2e-16
Cholesterol	3	8.36	10.77	2.4e-6
Residuals	128			

581

	Estimate	SE	t	p
β_0 (Intercept)	40.18	0.16	253.75	<2e-16
β_t (-z ; slope)	-2.35	0.08	-30.10	<2e-16
β_C (Cholesterol : 2.5 $\mu\text{g mg C}^{-1}$)	0.43	0.14	3.01	0.00324
(Cholesterol : 5 $\mu\text{g mg C}^{-1}$)	0.56	0.14	3.58	0.00018
(Cholesterol : 8 $\mu\text{g mg C}^{-1}$)	0.70	0.12	5.62	1.14e-07

582

583

584 **Figure legends**

585 **Figure 1: (A)** Thermal death time curves (TDT) predict how heat tolerance varies with the
586 intensity and duration of the thermal stress and are characterized by CT_{max} and z (see *eq. 2*;
587 figure adapted from Peralta-Maraver and Rezende, 2020) **(B)** Thermal death time curves for
588 *D. magna* as function of cholesterol supply in the diet. The color code and the effect of food
589 quality on CT_{max} ($\pm 95\%$ CI) are displayed in the inset plots. Time is shown at the log10 scale.

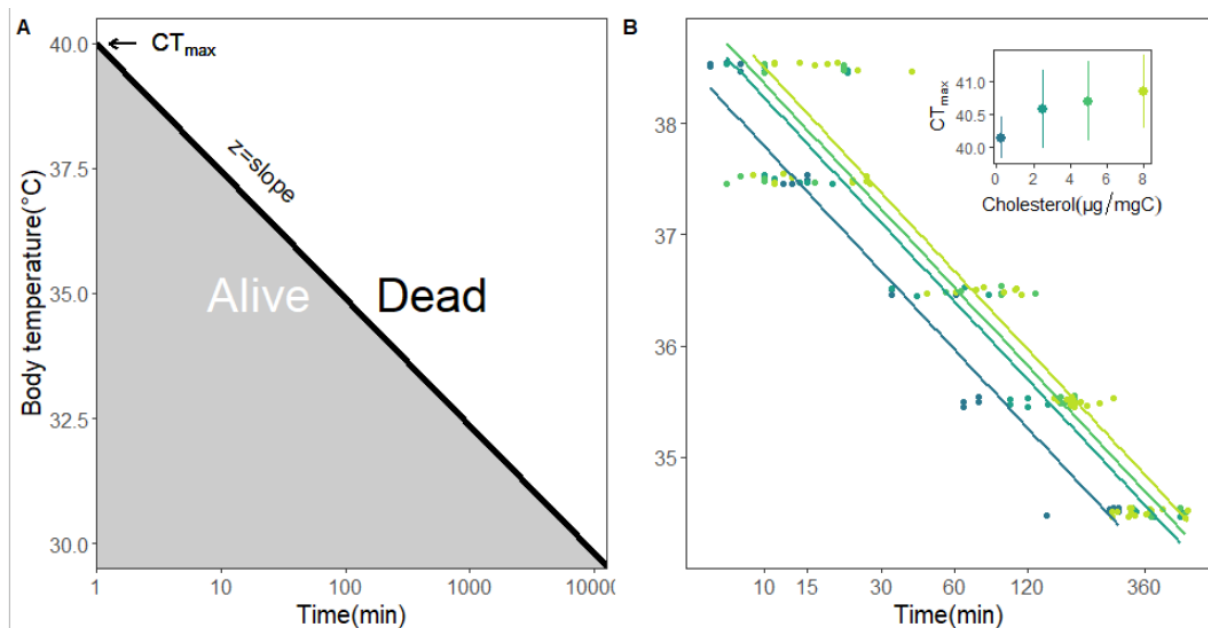
590 **Figure 2:** Predicted versus observed heat tolerance under constant temperatures for *D. magna*
591 as a function of cholesterol supply.

592 **Figure 3: (A)** Average heatwave intensity and duration for northern temperate shallow (<
593 5m) lakes under historical and future climate forcing (data from Woolway et al., 2021).
594 Intensity is defined as the increase in average daily surface temperature relative to the lake
595 climatology **(B)** Simulated temperature time-series when adding diurnal fluctuations to (A)
596 and assuming an 8°C daily cycling range as found on average in small lakes (Woolway et al.,
597 2016) **(C)** Boxplots of bootstrapped (n=100) predicted cumulative survival of *D. magna* to
598 simulated historical and future heatwaves (i.e. the times series shown in B) as a function of
599 cholesterol supply. The $\pm 95\%$ CI are given in the text (*see Results*)

600

601 **Figures**

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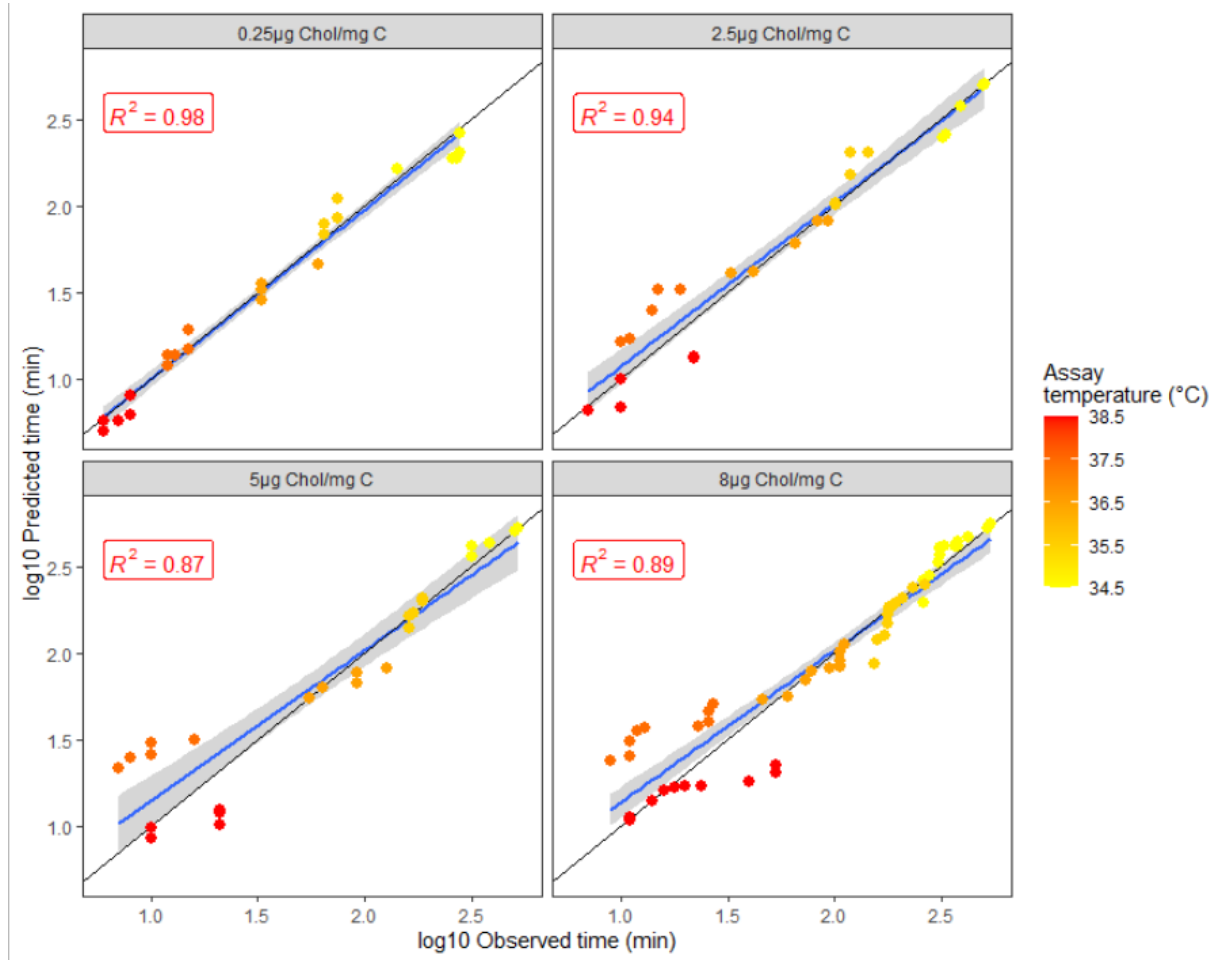


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604 **Figure 1**

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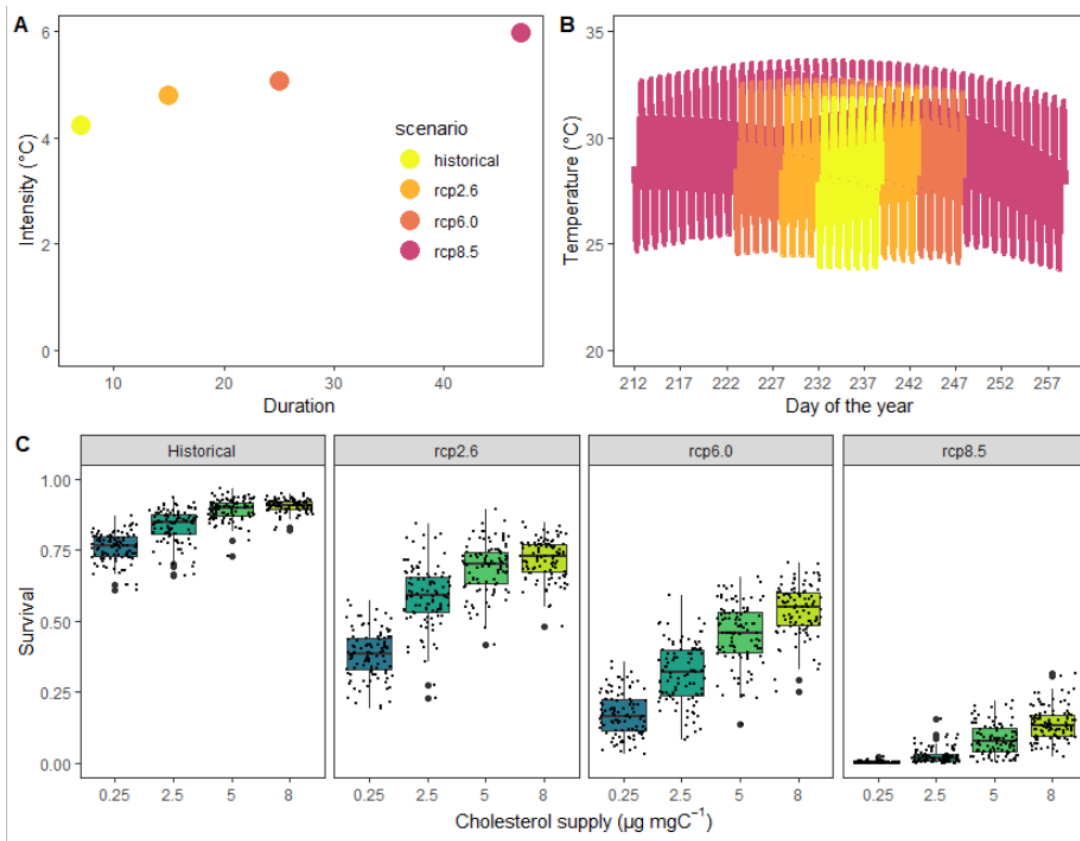
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608 **Figure 2**

609



610 **Figure 3**