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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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Dietary sterol availability modulates *Daphnia*'s heat tolerance

Apostolos-Manuel Koussoroplis^{1,4}, Erik Sperfeld², Sylvain Pincebourde³, Alexandre Bec¹, Alexander Wacker^{2,4}

Author affiliations:

¹ Université Clermont Auvergne, CNRS, LMGE, F-63000 Clermont–Ferrand, France.

² University of Greifswald, Zoological Institute and Museum, Loitzer Str. 26, 17489 Greifswald, Germany

³ Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS - Université de Tours, 37200 Tours, France

⁴ University of Potsdam, Institute of Biochemistry and Biology, Am Neuen Palais 10, 14469 Potsdam, Germany

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

Abstract (max 350 words)

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

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

Introduction

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

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

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

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

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

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

Methods

Theoretical framework

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

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

Which can also be rearranged as:

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

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

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

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

$$\frac{t_{imm,2}}{t_{imm,1}} = 10^{\frac{-(T_2-T_1)}{z}} \quad \text{eq. (3)}$$

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

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

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

Organism culture and nutritional conditioning

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

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

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

Thermal tolerance assays

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

Data analysis

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

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

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

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

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

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

Predicting survival under climate change-induced heat waves

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

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

Results

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

Acknowledgements:

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

Conflict of interest:

None

Author contributions:

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

Data availability statement

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

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Tables

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

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			

	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 μg mg C^{-1})	0.43	0.14	3.01	0.00324
(Cholesterol : 5 μg mg C^{-1})	0.56	0.14	3.58	0.00018
(Cholesterol : 8 μg mg C^{-1})	0.70	0.12	5.62	1.14e-07

Figure legends

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

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

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

Figures

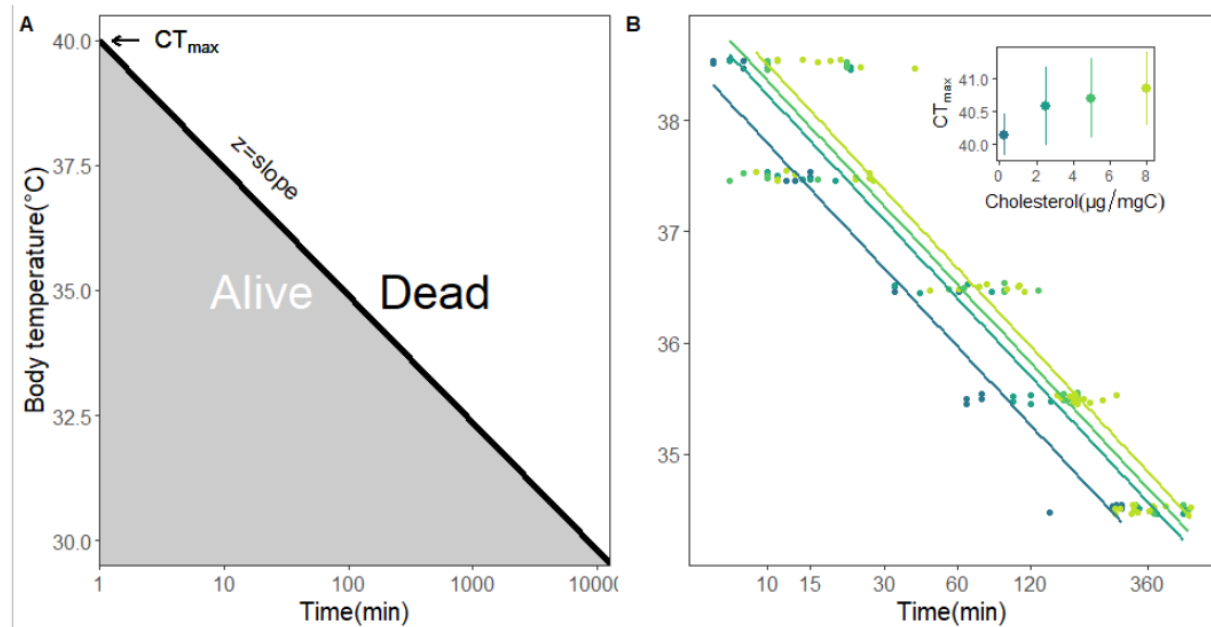
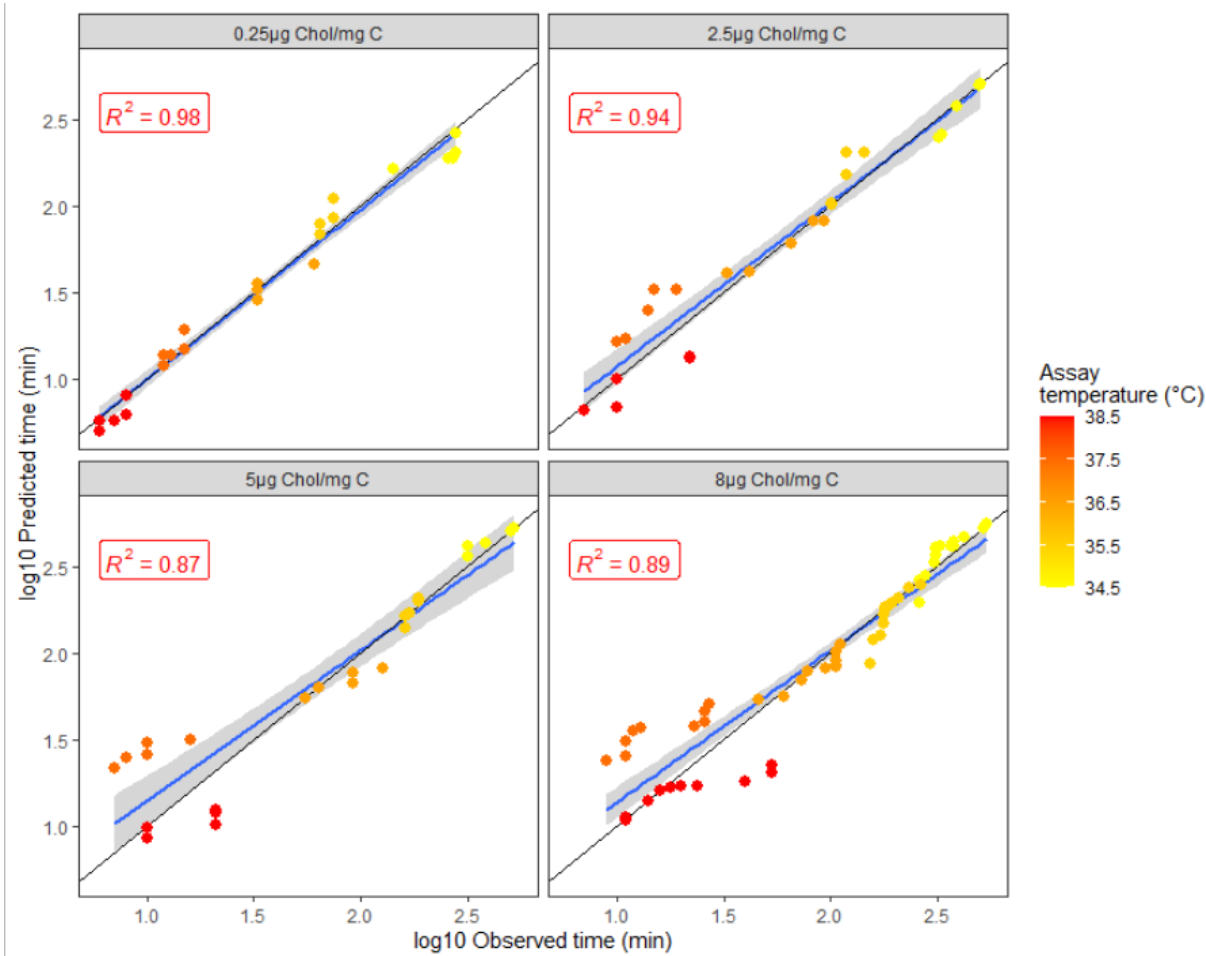


Figure 1

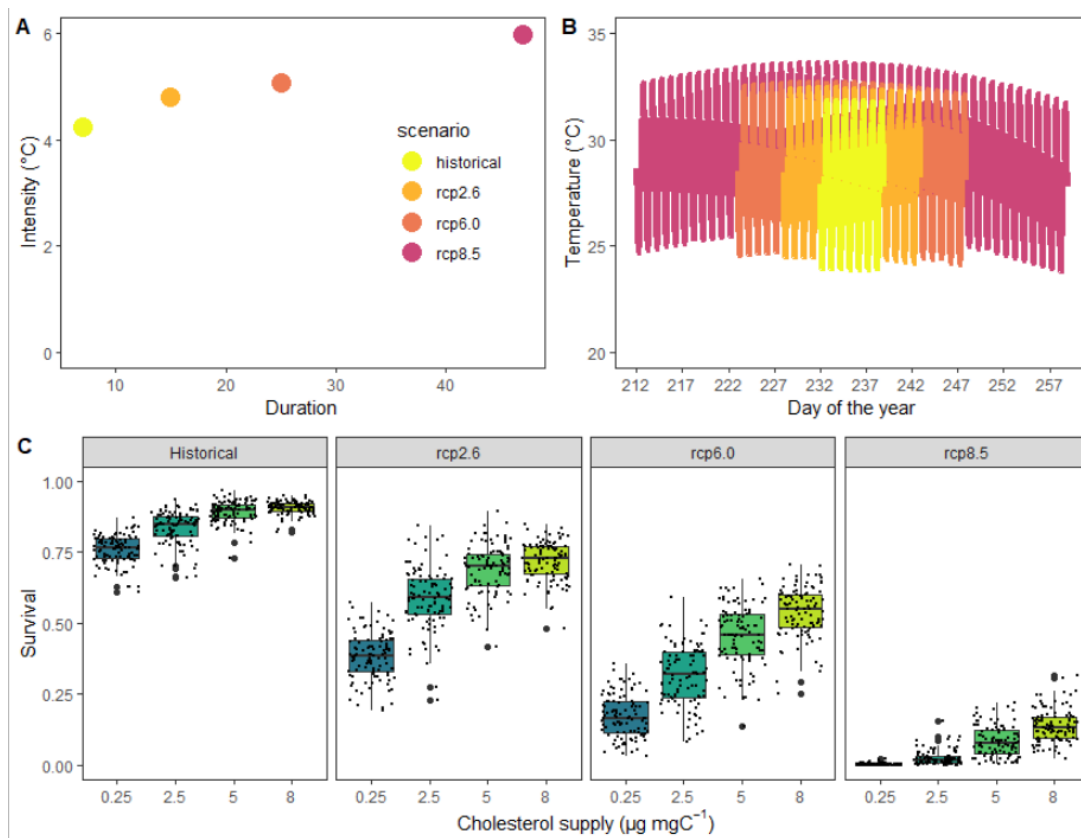
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608 **Figure 2**

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610 **Figure 3**