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Effects of temperature and dietary sterol availability on growth and cholesterol allocation of the aquatic keystone species *Daphnia*

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SUMMARY

Enhanced water temperatures promote the occurrence of cyanobacterial blooms, which may be detrimental to aquatic herbivores. Especially, the often-dominant crustaceans could be negatively affected because cyanobacteria are deficient in phytosterols, which are required by the crustaceans to form the membrane component cholesterol, which in turn plays a role in thermal adaptation. Here, we determined the influence of temperature on growth, reproduction and the allocation of dietary sterol into somatic tissues and eggs of the keystone species *Daphnia magna* raised along a dietary cholesterol gradient. Mass-specific growth rates of *D. magna* increased with the increasing availability of dietary cholesterol up to an incipient limiting level, which increased with increasing temperature. This indicates a higher demand for cholesterol for growth at higher temperatures and may explain the consistently smaller clutch sizes of reproducing females at the highest temperature. The cholesterol content of the individuals increased with increasing dietary cholesterol; this increase was enhanced at higher temperatures, indicating a higher demand for cholesterol for tissues and probably specifically for membranes. Surprisingly, the daphnids showed different allocation strategies with regard to temperature and dietary sterol availability. The cholesterol content of eggs was enhanced at higher temperature, which suggested that females allocate more cholesterol to their offspring, presumably to ensure sufficient egg development. When dietary cholesterol was limiting, however, females did not allocate more cholesterol to their eggs. Our data suggest that during cyanobacterial blooms, a potential dietary sterol limitation of *Daphnia* can be intensified at higher water temperatures, which can occur with global warming.

Key words: allocation strategy, cholesterol, growth, reproduction, temperature adaptation, zooplankton.

INTRODUCTION

Cholesterol is the predominant sterol in animals (Goad, 1981) and an indispensable structural component of their plasma membranes. In biological cell membranes, cholesterol, along with specific fatty acids, regulates the function of membrane-bound proteins and plays a role in thermal adaptation (Crockett, 1998; Hochachka and Somero, 2002). Furthermore, cholesterol serves as a precursor for multiple steroid hormones, e.g. ecdysteroids in arthropods, which are involved in the moulting process (Goad, 1981; Grieneisen, 1994; Martin-Creuzburg et al., 2007). Arthropods lack the ability to synthesize cholesterol and other sterols *de novo* and therefore have to acquire these essential compounds from their diet (Svoboda and Thompson, 1985; Martin-Creuzburg and Von Elert, 2009). Unlike carnivorous arthropods, herbivorous arthropods lack cholesterol in their diet and have to metabolize dietary phytosterols to form cholesterol to meet their requirements for growth and reproduction (Svoboda and Thompson, 1985; Martin-Creuzburg and Von Elert, 2004).

In the pelagic environment of aquatic ecosystems, there is a seasonal succession of suspended algal and cyanobacterial species (Sommer et al., 1986; Sommer, 1989); these species can vary greatly in their elemental and biochemical composition (e.g. Ahlgren et al., 1997; Kreeger et al., 1997; Wacker and Von Elert, 2001; Wacker and Von Elert, 2004). Thus, one can assume that the amount and composition of dietary phytosterols in the pelagic environment vary over time. For example, cyanobacteria usually lack sterols (Volkman, 2003; Volkman, 2005; Summons et al., 2006); therefore, during cyanobacterial blooms, the lack of dietary sterols might limit growth, reproduction and development of aquatic herbivorous arthropods, which mainly consist of suspension-feeding crustaceans. Indeed, laboratory experiments have shown that the availability of dietary sterols (cholesterol, phytosterols) has serious consequences for various life history traits of the aquatic herbivorous arthropod *Daphnia* (Von Elert et al., 2003; Martin-Creuzburg et al., 2005; Martin-Creuzburg et al., 2009). This keystone species often provides an important link for the transfer of carbon from primary producers to higher trophic levels (Sterner and Hessen, 1994; Gaedke and Straile, 1998).

The effect of sterol limitation observed in controlled laboratory experiments, however, might differ from the effect in the natural environment because of prevailing environmental conditions and other factors that influence the growth and reproduction of *Daphnia*. For instance, temperature strongly influences the metabolic rate of these ectothermic species (Dawidowicz and Loose, 1992), and *Daphnia* grows and reproduces faster at higher temperature (Giebelhausen and Lampert, 2001; Rinke and Petzoldt, 2003). In contrast to this promoting effect of temperature on the metabolic activity, increasing temperature could also hamper the growth or development of *Daphnia* owing to changed physiological demands for cholesterol. Cholesterol plays an important role in the adaptation to temperature because of its membrane-stabilizing effects (Robertson and Hazel, 1997). These effects may be greater at higher temperatures, where a higher loss of the membrane's static order occurs (Robertson and Hazel, 1995). Thus, with increasing

temperature, more cholesterol may be required to maintain suitable membrane properties, and this temperature-dependent cholesterol effect on membrane functionality may lead to different sterol requirements for *Daphnia* between different temperatures. The sterol requirements of *Daphnia* and other species might not only be affected by rapid changes in water temperature but also by slow changes, such as those that occur during global warming. Such long-term trends of increasing water temperature have been predicted for shallow lakes because of the very tight coupling between air and water temperatures (Mooij et al., 2008). Enhanced sterol requirements with increasing temperature may influence both ecosystem processes and community structures because the outcome of species competitions could differ at different temperatures. Additionally, increasing temperature favours the growth of cyanobacteria (Jöhnk et al., 2008) and enhances the possibility of harmful cyanobacterial blooms, which threaten many aquatic ecosystems (Paerl and Huisman, 2008). The promoting effect of increasing temperature on the occurrence of cyanobacteria may intensify the sterol limitation of zooplankton and therefore has a positive indirect effect on these bacteria, leading to a faster development of potentially harmful blooms.

From an evolutionary point of view, an individual should allocate a higher fraction of a growth-limiting resource to its offspring to increase the chances of offspring survival or to improve the offspring's initial growth. Considering its own survival, an individual should increase its fitness by a higher allocation of limiting resources to a reduced number of offspring (Stearns, 1992). This has been demonstrated for *Daphnia* in terms of food quantity (Guisande and Gliwicz, 1992), but little is known about its allocation strategy in terms of food quality (Becker and Boersma, 2005; Wacker and Martin-Creuzburg, 2007). Based on the arguments presented above, we would expect a temperature-dependent allocation strategy for essential membrane-bound lipids such as cholesterol.

Here, we assessed the effects of temperature and dietary sterol availability, as well as their potential interplay, on the performance and allocation strategies of *Daphnia magna*. To examine how sterol limitation of *D. magna* is affected by temperature, we determined the growth and reproduction response of the animals to increased dietary cholesterol at different temperatures. To assess potentially different cholesterol demands at different temperatures, we determined the amounts of cholesterol in the tissue of individuals. The temperature-dependent cholesterol content of *D. magna* was measured separately for eggs and somatic tissues to investigate potential allocation strategies (i.e. whether a temperature adaptation exists and whether the cholesterol content in the eggs is adjusted to the temperature-dependent demand of the offspring).

MATERIALS AND METHODS

Cultivation of organisms

The stock culture of a clone of *Daphnia magna* Straus (Lampert, 1991) was maintained in filtered lake water (0.2 µm pore-sized membrane filter) with saturating concentrations of the green alga *Scenedesmus obliquus* (SAG 276-3a, culture collection Göttingen, Germany) at 20°C. For growth experiments, the edible, non-toxic and sterol-free cyanobacterium *Synechococcus elongatus* (SAG 89.79) was used (Lampert, 1977; Lampert, 1981; Martin-Creuzburg et al., 2008). *S. obliquus* and *S. elongatus* were cultured semi-continuously (dilution rate: 0.4 day^{-1} and 0.2 day^{-1}, respectively) in aerated 2-liter flasks containing modified WC medium with vitamins (Guillard, 1975) at 20°C and an illumination of 120 µmol m^{-2} s^{-1} and 40 µmol m^{-2} s^{-1}, respectively, with a 16 h:8 h light:dark cycle.

Preparation of food

Cholesterol-containing liposomes used as food supplements were prepared according to a previously successfully tested method (Martin-Creuzburg et al., 2008). The liposome stock solution was prepared from 3 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and 7 mg 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC; Lipoid, Ludwigshafen, Germany) dissolved in an aliquot of ethanol. 3.33 mg of cholesterol (Sigma, Steinheim, Germany) was added from a cholesterol solution in ethanol. The mixture was dried using a rotary evaporator and dissolved in 10 ml buffer (20 mmol l^{-1} NaP_i, 150 mmol l^{-1} NaCl, pH 7.0). Afterwards, the liposome suspension was sonicated in an ultrasonic bath for 30 s, and excess free cholesterol was removed by washing the liposomes in fresh buffer using an ultra-speed centrifuge (15,000 ***g***, 360 min, 4°C). Liposome suspensions were stored at –20°C until preparation of experimental food treatments.

During experiments, carbon concentrations of cyanobacterial food suspensions (2 mg C l^{-1}) were estimated from photometric light extinction (800 nm) using previously determined carbon-extinction equations. Aliquots of *S. elongatus* were filtered onto precombusted glass fibre filters (Whatman GF/F, 25 mm) and dried for later analysis of particulate organic carbon using a carbon analyser (HighTOC+N, Elementar, Hanau, Germany). The cholesterol content of liposomes added to food suspensions was determined from subsamples during the experiment; 1 µl of the liposome suspension contained 0.35±0.06 µg cholesterol (mean ± s.d., N=9) and the mean diameter of liposomes was 2.3±1.0 µm (N=15,322; determined using a CASY particle counter, Schärfesystem, Germany). To determine the dietary cholesterol level, the amount of cholesterol added by liposomes to food suspensions was related to the measured carbon concentrations of *S. elongatus*. Direct measurement of the cholesterol content of experimental food suspensions was not necessary because liposomes are very stable during 24 h of usage (E.S. and A.W., unpublished).

Experimental design

Fourth-clutch juveniles of *D. magna*, hatched within 12 h in filtered lake water containing *S. elongatus* as food, were used for the experiments. To examine temperature dependencies, the experiments were run at 15, 20 and 25°C in vessels with 300 ml filtered lake water and 2 mg C l^{-1} of *S. elongatus*. To test for cholesterol limitation of *Daphnia* growth, we generated a gradient of dietary cholesterol level by adding different amounts of cholesterol-containing liposomes to sterol-free *S. elongatus*. To obtain a narrow cholesterol gradient in the diet, we applied 19 cholesterol concentrations (ranging from 0 to 53 µg cholesterol per mg dietary carbon) per temperature without replication. Throughout the experiments, daphnids were transferred daily into vessels with renewed food suspensions.

The experiments were terminated a few hours after the majority of females provided with high dietary cholesterol levels had released their first clutch into the brood pouch. This was dependent on the temperature and occurred after 8.5, 6 and 5 days at 15, 20 and 25°C, respectively. The eggs of the first clutch were separated from the somatic tissue of a female by gently blowing them out of the brood pouch with a drawn-out glass Pasteur pipette. Subsamples of both the body of females (soma) and eggs were used for the determination of dry mass or for the analysis of cholesterol content. For determination of dry mass, 2–9 bodies or 4–24 eggs were dried in pre-weighed aluminium boats for 48 h at 50°C and weighed on an electronic balance (±1 µg; CP2P, Sartorius, Goettingen, Germany). The juvenile mass-specific growth rates (g) were determined as the

increase in total dry mass from the beginning (DM_0) to the end of an experiment (DM_t) using the equation $g=(\ln DM_t-\ln DM_0)/t$, where t is the duration of the experiments in days.

Chemical analysis

The cholesterol content of *D. magna* was determined in at least three bodies or 10–50 eggs. The daphnids or eggs were transferred to dichloromethane/methanol (2:1, v:v), sonicated for 3 min and stored under nitrogen at –20°C for later analysis. 5-α-Cholestan (Sigma) was used as an internal standard, and lipids were extracted twice with dichloromethane/methanol (2:1, v:v). The particles were removed by centrifugation (1730 ***g*** for 5 min), and the supernatant was evaporated to dryness under nitrogen. The evaporated sample was saponified for 60 min at 70°C with 0.2 mol l^{-1} methanolic KOH and subsequently extracted three times with *iso*-hexane. The neutral lipids were partitioned into *iso*-hexane:diethyl ether (9:1, v:v), and this cholesterol-containing fraction was evaporated to dryness under nitrogen and resuspended in 20–50 µl of *iso*-hexane. Cholesterol was identified and quantified using a gas chromatograph (6890 N, Agilent Technologies, Santa Clara, CA, USA) with a DB-5 ms capillary column as described previously (Wacker and Martin-Creuzburg, 2007). 1 µl of the sample was injected with the split setting of 5:1 (i.e. one-fifth of the sample gets into the capillary column); cholesterol was identified by comparison of the retention times with authentic cholesterol and quantified using the internal standard (5-α-cholestan).

The amount of cholesterol in *D. magna* was related to the carbon content of somatic tissues and eggs instead of dry mass to diminish the effect of the carapace, which likely has a low cholesterol content and a relatively high mineral content (Hessen and Rukke, 2000). The mean carbon content (± s.d.) of somatic tissues (N=18) was 0.41±0.02 µg µg^{-1} dry mass and that of the eggs (N=22) was 0.50±0.06 µg µg^{-1} dry mass; the carbon content was determined from random subsamples at each of the temperatures using a carbon analyser (HighTOC+N, Elementar, Hanau, Germany). Thus, somatic tissues contained 18% fewer carbon per dry mass than egg tissue.

Statistical analysis

We fit linear regression models with segmented relationships (Muggeo, 2003) using the somatic growth rates (g) at the three temperatures, dependent on the dietary cholesterol level (Ch). The incipient limiting level of growth was estimated by determining the break-point between cholesterol-limited growth rates (g_{lim}) and maximal growth rates (g_{max}) for each temperature. A higher break-point (i.e. incipient limiting level) indicates a higher cholesterol demand. Slopes of g_{lim} and break-points were compared on the basis of their 95% confidence intervals to assess differences among temperatures.

Life history parameters (growth rate, clutch size, and mass of the individual, their somatic tissue or eggs) and biochemical variables (cholesterol content of the whole individual, their somatic tissue or eggs) were analysed using an analysis of covariance (ANCOVA), with dietary cholesterol level as the covariate and temperature as the factor. To meet assumptions for ANCOVA of the biochemical variables, the dietary cholesterol level was $\log_{10}$-transformed. This transformation also adjusts for the increasing intervals between the dietary cholesterol levels and prevents an overestimation of data at high dietary cholesterol levels.

Changes of life history parameters or biochemical variables relating to temperature were compared with Tukey HSD *post hoc* tests only if there was no trend for an interaction between dietary cholesterol level and temperature (P>0.2). An interaction indicated different slopes of the response variable along the covariate (dietary cholesterol level) between at least two factor levels (Underwood, 2001), thereby disturbing a correct interpretation of the temperature effect in the present study. We conservatively used an interaction of P>0.2 as the break-off criterion mentioned above so that we could be sure that there is no hidden difference between the slopes at different temperatures. Below that value, temperatures were compared pair-wise using individual ANCOVAs to clarify at which temperatures the slopes were different. This resulted in three comparisons per response variable (e.g. cholesterol content of the whole individual, their somatic tissue or eggs). If there was no difference between the slopes of the response variable at two temperatures, the elevations were directly compared.

RESULTS

Growth and fecundity

At all three temperatures, the mass-specific growth of *D. magna* increased with increasing availability of dietary cholesterol up to an incipient limiting level of 7–9 µg mg^{-1} C (Fig. 1A), which was determined by the break-point of the segmented linear regressions (Table 1). The break-point, i.e. the offset of growth limitation of *D. magna* by cholesterol, increased with increasing temperature (Table 1). The marginal interaction at cholesterol-limited growth rates (g_{lim}) (Table 2) indicated that the effect of temperature may

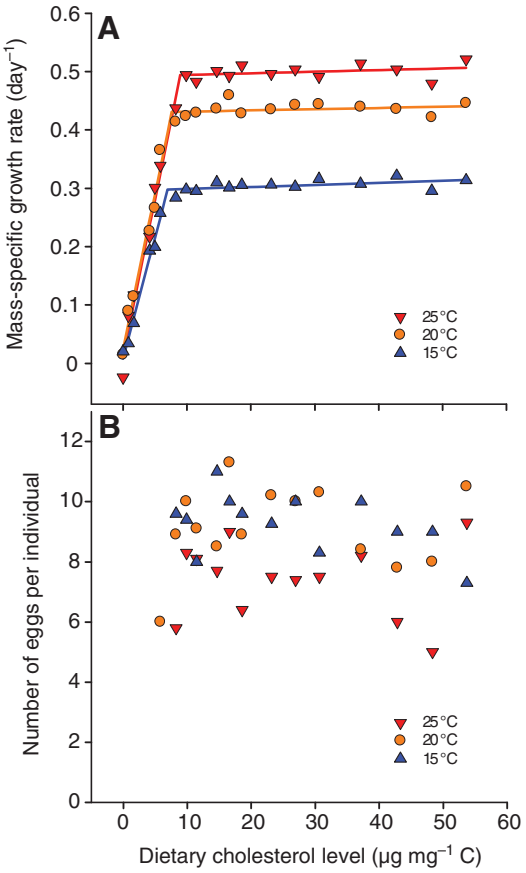

Fig. 1. Mass-specific growth (A) and fecundity (B) of *D. magna* in response to dietary cholesterol levels at different temperatures. The regression lines in A indicate linear regression models with segmented relationships (Muggeo, 2003) (r^2=0.99 at all three temperatures). At low dietary cholesterol levels, females did not produce eggs until the end of experiments.

Table 1. Break-points and slopes of the functional response of growth to dietary sterol availability calculated by linear regression models with segmented relationships between mass-specific growth rates of *D. magna* at different temperatures and dietary cholesterol levels (μg mg^{-1} C)

Temperature	Break-point	Slope
15°C	6.98 (6.49–7.46)[a]	0.042 (0.038–0.045)[a]
20°C	7.62 (6.97–8.27)[a]	0.053 (0.047–0.059)[b]
25°C	8.96 (8.33–9.59)[b]	0.054 (0.049–0.059)[b]

Slopes refer to the first segments of regressions at low dietary cholesterol levels (cholesterol-limited growth, g_{lim}), including the break-point. 95% confidence intervals are given in parentheses, and identical superscript letters indicate estimates that have overlapping confidence levels.

originate from the lower slope of growth rates at 15°C compared with the slopes at 20 and 25°C, which were nearly identical (Table 1). The maximum growth rates strongly increased with increasing temperature (Table 2) (Tukey's HSD: comparisons among all temperatures, P<0.0001). Close to the incipient limiting level, the females produced eggs at all three temperatures, and their fecundity (clutch size) was highly variable and showed no clear response to the dietary cholesterol level (Fig. 1B; Table 2). Only temperature had an effect on clutch size; clutches were smaller at 25°C than at 15 and 20°C, which were of similar size (Tukey's HSD, 25°C *vs* 15 or 20°C, P<0.003; 15°C *vs* 20°C, P=0.96).

Carbon-related masses of individuals, somatic tissues and eggs

The carbon-related masses of individuals (somatic tissues + eggs) at maximum growth rates were only affected by temperature (Table 2); animals cultured at 15 and 20°C were heavier than animals cultured at 25°C (Fig. 2A) (Tukey's HSD, 25°C *vs* 15 or 20°C, P<0.0001; 15°C *vs* 20°C, P=0.31). This was similar but less pronounced for somatic tissues of females (Fig. 2A; Table 2) (Tukey's HSD, 25°C *vs* 15 or 20°C, P<0.02; 15°C *vs* 20°C, P=0.51). The carbon-related individual egg masses were independent of the dietary cholesterol level and of temperature (Table 2, egg mass) and were about 2.12±0.22 μg carbon (mean ± s.d., N=40). However, there was a trend of decreasing individual egg mass with increasing temperature (Fig. 2B) (regression, F=2.94, t=1.72, d.f.=38, P=0.094).

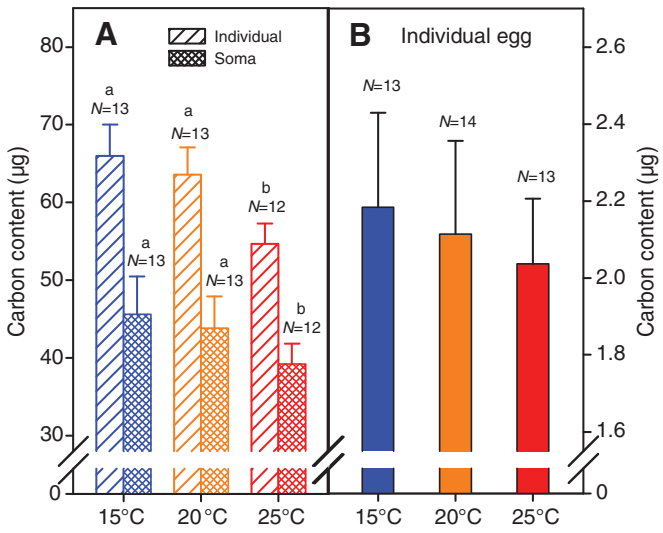

Fig. 2. Mean carbon-related mass of *D. magna* females with eggs of the first clutch (individual) and with eggs removed (soma) at maximum growth rates (A) and mean carbon-related individual egg mass (B) at different temperatures. Error bars indicate s.d. for the number of observations indicated above them. Identical letters denote non-significant differences among temperature treatments (Tukey's HSD, P<0.05).

Cholesterol content of individuals, somatic tissues and eggs

Increasing dietary cholesterol level resulted in increased cholesterol content of the whole individual (somatic tissue + eggs) at all three temperatures (Fig. 3A–C; Table 3). The slope of this relationship was higher at elevated temperatures (Table 3, significant interaction; Table 4), which indicated higher incorporation of cholesterol into tissues of animals grown at higher temperatures with increasing dietary cholesterol. At very low dietary cholesterol levels, the higher cholesterol content of *D. magna* relative to the diet indicated an accumulation of cholesterol, whereas at high dietary cholesterol levels, the lower cholesterol content of *D. magna* relative to the diet indicated stoichiometric excretion or no assimilation of cholesterol (Fig. 3A–C).

Interestingly, the cholesterol content of eggs was higher than that of somatic tissues (Fig. 3D–F) (mean of all three temperatures ± s.d.

Table 2. Statistical results of ANCOVA for changes in life history parameters of *D. magna* depending on the covariate dietary cholesterol level (*Ch*) and the factor temperature (*T*)

	g_{lim}				g_{max}				Clutch size			
Factor	d.f.	*F*	*P*		d.f.	*F*	*P*		d.f.	*F*	*P*	
Ch	1,13	567.15	<0.0001	***	1,32	15.01	0.0005	***	1,34	0.53	0.4698	
T	2,13	8.62	0.0041	**	2,32	1008.30	<0.0001	***	2,34	9.13	0.0007	***
Ch×*T*	2,13	3.02	0.0836		2,32	0.12	0.8850		2,34	0.67	0.5202	
	$Mass_{max}$ individual				$Mass_{max}$ soma				Mass egg			
Factor	d.f.	*F*	*P*		d.f.	*F*	*P*		d.f.	*F*	*P*	
Ch	1,32	0.21	0.6479		1,32	2.76	0.1064		1,34	0.02	0.8855	
T	2,32	24.53	<0.0001	***	2,32	9.08	<0.0001	***	2,34	1.54	0.2282	
Ch×*T*	2,32	0.73	0.4915		2,32	0.88	0.4240		2,34	1.98	0.1530	

Life history parameters: *g*, mass-specific growth rate; clutch size; mass of an individual (somatic tissue + eggs of the first clutch) or its somatic tissue (soma = body of a female without eggs); and egg mass. Analyses of the mass-specific growth rates were separated in a region below (g_{lim}) and above (g_{max}) the incipient limiting level (determined by the break-point of the linear regression model with segmented relationships) of the functional response to dietary cholesterol levels. The carbon-related mass of individuals and their somatic tissues was analyzed at maximum growth rates (g_{max}).

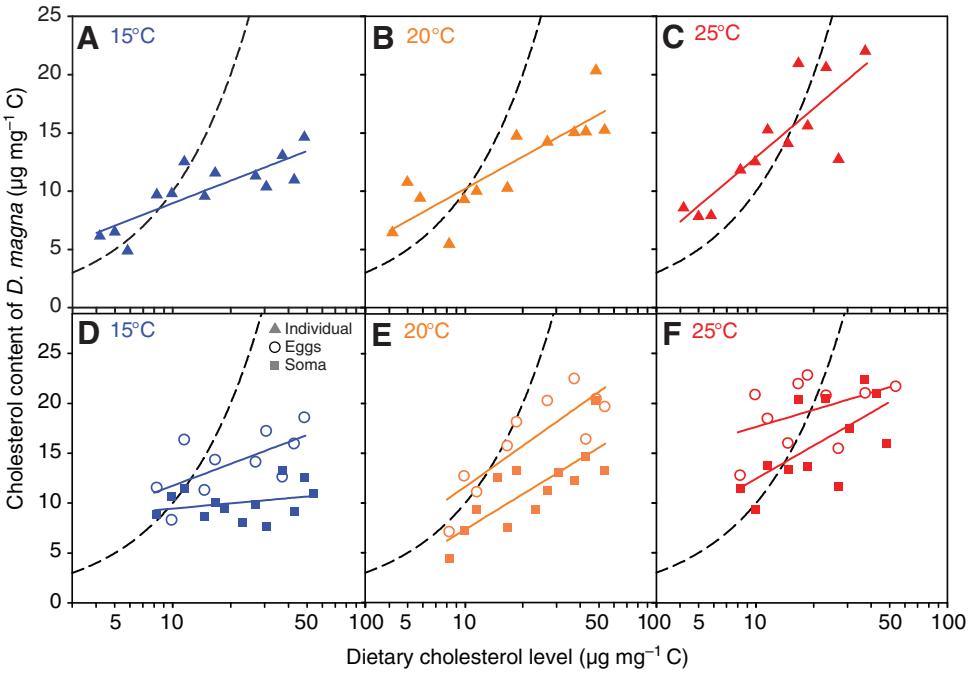

Fig. 3. Cholesterol content of *D. magna* in response to dietary cholesterol level for females with eggs of the first clutch (individual; A,B,C), with eggs removed (soma), and the eggs of the first clutch (D,E,F) at three different temperatures. Solid lines indicate simple linear regression models and dashed lines indicate the 1:1 ratio of the dietary cholesterol level and the cholesterol content of *D. magna*.

in µg cholesterol mg^{-1} carbon: eggs, 16.55±4.28, N=30; somatic tissue, 12.38±4.24, N=38; t-test, t=4.00, d.f.=66, P=0.0002). This outcome becomes even stronger if dry masses are used in the denominator instead of carbon-related masses (mean of all three temperatures ± s.d. in µg cholesterol mg^{-1} dry mass: eggs, 8.27±2.14; somatic tissue, 5.08±1.74; t-test, t=6.80, d.f.=66, P<0.0001).

At 15°C, the cholesterol content of the somatic tissue of *D. magna* did not significantly increase whereas at 20°C it increased with increasing dietary cholesterol level (Fig. 3D,E; Table 4) ($Ch\times T$, F=9.25, d.f.=1,22, P=0.006). Also, at 25°C the cholesterol content of the somatic tissue increased with increasing dietary cholesterol level, similar to that at 20°C (Fig. 3E,F; Table 4) ($Ch\times T$, F=0.02, d.f.=1,21, P=0.88), but was higher than at 20°C at a given dietary cholesterol level (temperature, F=15.67, d.f.=1,21, P=0.0007), which indicated again a higher demand of cholesterol in tissues of animals grown at higher temperatures. The cholesterol content of the eggs was highly variable and increased with increasing dietary cholesterol level at 15 and 20°C and tended to increase at 25°C (Tables 3 and 4). Furthermore, the cholesterol content of eggs at 15 and 20°C did not differ at a given dietary cholesterol level (temperature, F=2.90, d.f.=1,16, P=0.11), and both were significantly lower than the cholesterol content of eggs at 25°C (temperature, F>7.04, d.f.=1,16, P<0.018), which indicated an enhanced allocation of cholesterol into eggs at high temperature. In summary, our data showed a clear trend in an increased incorporation of cholesterol into somatic tissues and egg material of *D. magna* at elevated temperatures if dietary cholesterol was sufficiently available.

DISCUSSION

Our results demonstrated that temperature affects the growth limitation of *D. magna* by dietary sterol as well as the female's mass at first reproduction and the clutch size. The responses of the life history parameters indicated a higher demand for cholesterol at higher temperatures. This was confirmed by increasing amounts of cholesterol found in animals at higher temperatures when sufficient dietary cholesterol was available. Furthermore, we found that the cholesterol content in the eggs was higher than in the somatic tissue of the females, and the cholesterol content of both eggs and somatic tissues increased more or less with increasing temperature and increasing available dietary cholesterol. This enables predictions about the allocation strategy of *D. magna* for cholesterol in an environment varying in temperature and the availability of this essential nutrient. We will discuss this allocation strategy as well as the effects of temperature, dietary cholesterol availability and their potential interaction on life history parameters of this important species in the food web of pelagic freshwater ecosystems.

Physiological (metabolic) effect of temperature

Generally, the growth of organisms becomes faster with increasing temperature until a physiological optimum is reached. Above the

Table 3. Statistical results of ANCOVA for changes in the cholesterol content of whole individuals (somatic tissues + eggs of the first clutch), somatic tissues (soma = body of a female without eggs) and eggs of the first clutch, depending on the covariate dietary cholesterol level (Ch, $\log_{10}$-transformed) and the factor temperature (T)

	Individual				Soma				Eggs			
Factor	d.f.	F	P		d.f.	F	P		d.f.	F	P	
Ch ($\log_{10}$)	1,32	63.14	<0.0001	***	1,32	18.87	0.0001	***	1,24	19.63	0.0002	***
T	2,32	14.09	<0.0001	***	2,32	17.83	<0.0001	***	2,24	9.71	0.0008	***
$Ch\times T$	2,32	3.31	0.0494	*	2,32	3.71	0.0357	*	2,24	1.61	0.2213	

The d.f. for the analysis of eggs is lower because *D. magna* did not produce eggs at low dietary cholesterol levels.

Table 4. Slopes of the cholesterol content of *D. magna* (μg mg^{-1} C) as a function of dietary cholesterol level (μg mg^{-1} C, $\log_{10}$-transformed) for the whole individual ($slope_I$), the somatic tissue ($slope_S$) and the eggs of the first clutch ($slope_E$) at the three tested temperatures, calculated by simple linear regression models

Temperature	$Slope_I$	$Slope_S$	$Slope_E$
15°C	6.40 (3.52–9.28)[a]	1.73 (–2.31–5.77)[a]	7.27 (0.28–14.25)[a]
20°C	9.10 (5.39–12.82)[a,b]	11.74 (5.72–17.75)[b]	13.63 (6.65–20.60)[a]
25°C	13.90 (7.58–20.22)[b]	11.02 (1.69–20.35)[b]	5.70 (–3.86–15.25)[a]

95% confidence intervals are given in parentheses. A negative lower confidence interval indicates a non-significant slope. Identical letters in superscript indicate slopes that were not significantly different among temperatures after pair-wise comparisons using individual ANCOVAs (P<0.05).

optimal temperature, growth rapidly declines, giving the characteristic skewed curve of reaction norms of growth rates with temperature. The growth rate of *Daphnia* is the result of all underlying physiological rates that may depend differently on temperature (Angilletta et al., 2004). In our study, temperature was not expected to have a negative effect on the maximum growth rate because the optimal temperature for many clones of *D. magna* is above 25°C (Mitchell and Lampert, 2000). Accordingly, the maximum growth rate of our clone increased as the temperature increased from 15 to 25°C. Despite faster growth at the higher temperature, the individuals at 25°C were lighter and produced fewer eggs than the individuals at 15 and 20°C. This is in accordance with the 'temperature-size rule', which states that a reduction in environmental temperature causes an increase in size at maturity (Atkinson, 1996) and which has been observed in laboratory studies of many ectotherms (Atkinson, 1995). The smaller body of our animals at higher temperature might be responsible for the smaller clutch size because body size and clutch size of *Daphnia* are correlated at non-limiting food quantities (e.g. Lynch, 1980; Gliwicz and Boavida, 1996).

Interactive effect of temperature and cholesterol

We were interested to determine whether the different demands for cholesterol at the different temperatures cause changes in the life history parameters of the important freshwater herbivore *Daphnia*. Our results demonstrated that *D. magna* has an enhanced demand for cholesterol with increasing temperature.

The first evidence for the increased demand for cholesterol with increasing temperature is that the incipient limiting level for somatic growth, determined by the break-point of growth responses, increased with increasing temperature (Fig. 1A; Table 1). Therefore, the limitation of growth by cholesterol at higher temperature was offset at higher dietary cholesterol levels or, in other words, the saturation threshold of dietary cholesterol for growth of *D. magna* increased with increasing temperature. In a recent study examining the effects of temperature on the cholesterol content of several marine copepods, Hassett and Crockett investigated the growth response of one copepod (*Eurytemora affinis*) to the dietary cholesterol level at two temperatures (Hassett and Crockett, 2009). They also observed a higher saturation threshold at the higher temperature for this species raised at 6 and 25°C. In their experiments, the differences in the two saturation thresholds were more pronounced (4-fold; <0.05 and <0.2 μg cholesterol l^{-1} at 6 and 25°C, respectively) than in our experiments (1.3-fold for 15 up to 25°C) and may be a result of the larger temperature range in their experiment. Furthermore, our saturation thresholds for the somatic growth rates (break-points, 7–9 μg mg^{-1} C) at all three temperatures were somewhat higher than the thresholds given previously for two *Daphnia* species grown at 20°C [*D. galeata*, 2 μg mg^{-1} C (Von Elert et al., 2003); both *D. galeata* and *D. magna*: 5.4 μg mg^{-1} C (Martin-Creuzburg et al., 2005)]. The differences in all three studies with *Daphnia* may be caused by different calculation methods used for the determination of the saturation threshold or, more likely, by different methods used to produce the dietary sterol gradient and therefore by a possible varied availability of sterol for the consumer. The saturation thresholds described for the marine copepod (Hassett and Crockett, 2009) normalized on food quantity (at approximately 500 μg C l^{-1}, <0.1 and <0.4 μg cholesterol mg^{-1} C at 6 and 25°C, respectively) were far below the saturation thresholds for daphnids. One reason for these differences in the saturation thresholds could be that their stated carbon concentration was overestimated since values of carbon per cell from the literature were used. In particular, small differences in the actual cell size compared with values from the literature could result in large over- or under-estimates of carbon. Thus, the normalized values of saturation thresholds should be handled with caution. Another reason for the differences in saturation thresholds might be that the marine copepods and freshwater daphnids differ in their sterol requirements. This possibility is supported by an observation of Hassett and Crockett that *D. magna* showed a significantly higher cholesterol content than all of the examined marine copepods (Hassett and Crockett, 2009). Nevertheless, the cholesterol demand of our *D. magna* (break-points, 0.35–0.45% of dietary dry mass at a carbon-to-dry-mass conversion factor of 0.5) is within the range of sterol requirements determined for many other marine crustaceans [approximately 0.2–1% cholesterol per dry mass of the diet (Kanazawa, 2001)].

The second evidence for the increased demand of *D. magna* for cholesterol with increasing temperature is the increasing cholesterol content in tissues of animals with increasing temperature. However, a passive accumulation of cholesterol would be possible if one considers that cholesterol is not burned for energy regardless of need (Haines, 2001) in conjunction with the observation that the animals were lighter at higher temperatures, which might be caused by increased respiration rates. Our data illustrate that such a passive accumulation is rather unlikely because when excessive dietary cholesterol was available, the cholesterol content in the animals was lower at lower temperatures than at warmer temperatures. Thus, when sufficient dietary cholesterol was available, *D. magna* either actively excreted more cholesterol at lower temperatures or reduced the assimilation of cholesterol to maintain a suitable cholesterol concentration in the tissues. This indicates a temperature-dependent homeostatic regulation of the animal in terms of dietary sterols and suggests that animals incorporate more cholesterol in their membranes at higher temperatures to maintain appropriate membrane functions (Crockett, 1998).

By contrast, in a study that examined the cholesterol content of five marine copepods raised for 7–10 days at a high (16–25°C) and a low (6°C) temperature, no consistent pattern was observed (Hassett and Crockett, 2009). The cholesterol content of only one copepod species (*Calanus finmarchicus*) was higher at the higher temperature. No significant changes were observed in the other four species, not even in *Eurytemora affinis*, in which the saturation

threshold for growth increased with increasing temperature. This is in contrast to our results with *D. magna*, where both body content and the incipient limiting level increased with temperature.

Notably, in all of our results, the temperature-dependent differences were greater between 25 and 20°C than between 20 and 15°C. This could be caused simply by the exponential increase of metabolic rates with temperature (i.e. Q_{10} value). Alternatively, effects of cholesterol on membrane properties might also cause greater differences at higher temperatures for a number of reasons. Firstly, the membrane-stabilizing effects of cholesterol are greater at higher temperature because the static order of the phospholipid bilayer is highly disordered (Robertson and Hazel, 1995). Secondly, the influence of cholesterol on membrane properties is greater in membranes containing phospholipids with more saturated fatty acids (Crockett, 1998), which is usually the case at higher temperature (Pruitt, 1990). Thus, at higher temperatures, the membrane properties may be regulated to a greater extent by cholesterol whereas at lower temperatures, the major role is probably played by the fatty acid composition (i.e. the unsaturation index) of the phospholipids (Crockett, 1998).

Allocation of cholesterol into somatic tissues or eggs

When sufficient dietary cholesterol was available, *D. magna* increased the cholesterol content of its somatic tissue with increasing temperature probably because of the higher demand for cholesterol for the biochemical processes already mentioned. Interestingly, at a given dietary cholesterol level, the cholesterol content in eggs was higher than in somatic tissues, a pattern that has also been observed for a marine copepod species (Hassett and Crockett, 2009). However, adult animals allocate a certain proportion of their mass to the chitinous carapace, which has a relatively high mineral content (Hessen and Rukke, 2000) and probably a low cholesterol content since cholesterol is primarily localized in plasma membranes. The carapace can therefore add a low cholesterol fraction to the mass of the somatic tissue, compared with eggs, and thus the cholesterol content of the membranes could actually remain constant while the mass-specific cholesterol content differs between somatic tissues and eggs. The same issue pertains to the study of Hassett and Crockett (Hassett and Crockett, 2009), who observed higher protein-related cholesterol contents in *Calanus* eggs than in copepodites because the cuticle is composed of both protein and chitin. We reduced the influence of minerals, which probably vary between adults and eggs, by considering carbon-related cholesterol contents instead of dry-mass-related cholesterol contents. This led to an attenuation of the still significant difference between somatic tissues and eggs. A further reduction in this difference is possible by taking into account the remaining carbon of the carapace. For our data, the proportion of the carapace would have to exceed 28% of the dry mass of the animal's soma to contradict our statistical difference in cholesterol contents of somatic tissue and eggs. Moult masses can be used to estimate the percentage of the carapace to the mass of an animal. Estimates from regressions of dry moult mass to dry body mass at high food levels (Glazier and Calow, 1992) for the sizes of females observed in our study (soma, 70–140 μg dry mass) resulted in a portion of 10–12%. This is less than the 18% already considered by the conversion of dry mass to carbon-related masses in this study (see also Materials and methods section). Thus, in fact, females allocated more cholesterol into egg material since the animals possibly differed in their cholesterol demand at different life history stages, an issue that needs further examination.

Furthermore, we observed some consensuses between our results and the findings of a previous study examining the allocation of cholesterol in eggs and somatic tissues of *D. magna* (Wacker and Martin-Creuzburg, 2007). In this earlier study, the daphnids were raised on a sterol-rich diet (*Nannochloropsis limnetica*) and then fed sterol-free food (i.e. only *Synechococcus elongatus*). Their somatic cholesterol content declined but the cholesterol content of the eggs remained relatively constant at approximately 3.5 μg mg^{-1} dry mass (approximately 7 μg mg^{-1} C; for conversion factor, see Materials and methods in the present study). This is in accordance with the results of our current study, in which the lowest cholesterol content of eggs was about 7.1 μg mg^{-1} C, which suggests a threshold of minimum cholesterol content of eggs. The minimum cholesterol content of somatic tissues (i.e. the potential threshold) was close to that of eggs [7.3 μg mg^{-1} C (present study); approximately 3 μg mg^{-1} dry mass=7.3 μg mg^{-1} C (Wacker and Martin-Creuzburg, 2007); for conversion factor, see Materials and methods], which suggests a threshold of minimum cholesterol content for tissues of reproducing females. Moreover, although the threshold of minimum cholesterol content for an animal's tissue may increase with increasing temperature, higher resolutions are needed at low dietary cholesterol levels.

To maximize the fitness of an individual, it may be advantageous to allocate a larger fraction of cholesterol to offspring expected to hatch both with food of a low dietary cholesterol level and in a warmer environment. For example, *Daphnia* respond to low food quantity by increasing the allocation per offspring, i.e. by producing larger eggs with more protein, lipid and carbon, compared with eggs produced when high amounts of food are available (Guisande and Gliwicz, 1992). In the present study and in a previous study (Wacker and Martin-Creuzburg, 2007), eggs of females reared on food with insufficient dietary sterol did not have higher cholesterol contents than eggs of females fed on food with sufficient or high dietary sterol. Thus, *D. magna* seems to have a different allocation strategy for dietary cholesterol than for carbohydrates, proteins and other lipids. Specifically, with increasing dietary sterol limitation, a female may decrease the allocation of cholesterol to each egg to a minimum amount sufficient to ensure offspring development. Thus, a female can maintain a certain clutch size with decreasing dietary cholesterol until a minimum cholesterol level per egg is reached (according to the potential threshold for eggs described above). Afterwards, the clutch size may decrease with decreasing dietary sterol availability, as shown by a previous experiment (Martin-Creuzburg et al., 2005). Such a strategy would be advantageous if excessive cholesterol in the eggs does not strongly improve the survival of offspring in an environment with little dietary sterols. An argument for the latter hypothesis is the decreasing allocation of cholesterol per offspring with increasing cholesterol limitation found in the present study. This in turn may explain the decreasing growth rates of offspring fed on a sterol-free diet (only *S. elongatus*) with increasing maternal sterol limitation found in the previous study (Martin-Creuzburg et al., 2005). These differences in offspring growth after 6 days feeding on the sterol-free diet were very small (mass-specific growth rates: 0.04–0.1 day^{-1}) and did not reach growth rates sufficient for reproduction (i.e. approximately 0.3 day^{-1} within 6 days at 20°C), so that there were no substantial differences in fitness of offspring regardless of the cholesterol provided by the mothers.

With respect to temperature, our animals showed another allocation response. *Daphnia magna* increased the cholesterol content of the eggs at 25°C compared with the cholesterol content at lower temperatures and simultaneously reduced the clutch size. Although this would be a valuable allocation strategy, we cannot

determine whether the smaller clutch size is an adaptive response to different temperatures or an allometric constraint of the relationship between the mother's body size and the number of offspring. The latter possibility is supported by the finding that females at 25°C were lighter than females at lower temperatures. Nevertheless, our study clearly shows that *D. magna* is able to adjust the cholesterol content of its eggs relative to the temperature. This suggests that a certain cholesterol level in eggs is required for ontogenetic development of offspring hatching at a prevailing temperature.

Concluding remarks

The present study shows that for daphnids potentially suffering from sterol limitation in natural lakes or ponds, the limitation can be intensified by global warming because of the elevated temperature of water bodies. Therefore, global warming can increase the negative effect of the nutritional inadequacy of cyanobacteria for *Daphnia* and may lead, together with detrimental effects of toxicity and poor edibility (e.g. see Tillmanns et al., 2008; Martin-Creuzburg et al., 2008), to a weaker control of cyanobacterial blooms by herbivorous crustaceans. This indication adds to the growing body of indirect effects of how rising temperatures can promote the occurrence of cyanobacterial blooms and thus lead to a poorer water quality of eutrophic lakes in general (Paerl and Huisman, 2008; Jöhnk et al., 2008). In addition, an increased demand for dietary sterols with increasing temperature could have consequences for zooplankton in a broader context. Since the composition of phytosterols is highly variable among algal species (Volkman, 2003), crustaceans can also be limited if the algae present contain a large amount of dietary sterols not suitable for conversion to cholesterol (Martin-Creuzburg and Von Elert, 2004). Thus, at elevated temperatures, planktonic crustaceans can suffer from a lack of suitable dietary sterols, which may, in addition to e.g. minerals (Sterner and Elser, 2002), fatty acids (Becker and Boersma, 2005) and even more complex ecophysiological links between mixotrophs and consumers (Wacker and Weithoff, 2009), reduce the trophic transfer efficiency of energy from primary production to higher consumers.

From an evolutionary point of view, *D. magna* feeding on diets with insufficient cholesterol levels did not, contrary to expectations, enhance the cholesterol content of eggs, which would have been advantageous for the offspring if they hatched in an environment lacking this essential nutrient. However, we found that females allocate more cholesterol to eggs at higher temperatures, which indicates a higher evolutionary pressure on the development of eggs (up to neonates) than for the growth period after the release from the brood chamber. This suggests that the temperature-mediated sterol limitation has stronger effects on the population level than on the individual level. Consequently, the higher sterol demand with increasing temperature, not only for tissues of mothers but also for the offspring, may lead to a lower number of offspring and thus to decreasing population growth rates.

LIST OF ABBREVIATIONS

Ch	dietary cholesterol level
g	mass-specific growth rate
g_{lim}	cholesterol-limited growth rates
g_{max}	maximal growth rates
T	temperature

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