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RESEARCH ARTICLE

Daphnia's dilemma: adjustment of carbon budgets in the face of food and cholesterol limitation

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ABSTRACT

We studied the carbon (C) metabolism in *Daphnia* when the amount of C (food quantity) and/or the content of biochemical nutrients (food quality) was limiting. Growth performances and C budgets of *Daphnia magna* (assimilation, faeces egestion, excretion and respiration measured by [^{14}C]-tracing) were analysed when animals were raised on different food quantities and concentrations of cholesterol, an essential biochemical food compound. Cholesterol is of special interest because it not only acts as limiting nutrient but also contributes to the overall C pool of the animals. As the tissue cholesterol concentration in *Daphnia* is quite low, we hypothesized the selective exclusion of cholesterol from C budgeting and tested this using radiolabelled cholesterol. Somatic growth rates of *D. magna* were highest at high quantity and quality and were reduced to a moderate value if either the food quantity or the cholesterol concentration was low. Growth was lowest at low food quantity and quality. The measurements of C budgets revealed high regulative response to low food quality at high food quantity only. Here, low dietary cholesterol caused bulk C assimilation efficiency (AE) to decrease and assimilated (excess) C to be increasingly respired. Additionally, *Daphnia* enhanced efficient adjustment of C budgets when facing cholesterol limitation by (1) increasing the AE of the cholesterol itself and (2) not changing cholesterol respiration, which was still not detectable. In contrast, at low food quantity, *Daphnia* is unable to adjust for low food quality, emphasizing that food limitation could overrule food quality effects.

KEY WORDS: Biochemical limitation, Carbon budgets, Zooplankton, Carbon pathway, Food quality, Food quantity

INTRODUCTION

Information on the flow of energy and nutrients is necessary for the understanding of the individual performance of consumers, trophic interactions and regulation in food webs (Andersen, 1997; Gaedke et al., 2002). In aquatic ecosystems especially there are two major issues controlling the interaction between primary producers and primary consumers: first, the amount of energy [e.g. in terms of carbon (C)] supplied by the phytoplankton community (food quantity) and, second, the content of nutrients (e.g. minerals or essential biochemicals) in the algae (food quality). Cladocerans, as predominant filter-feeding zooplankton, are limited by energy availability because of low C concentrations, e.g. in the clear-water phase in spring (Sommer et al., 1986; Jeppesen et al., 1999) or due to reduced ingestibility (e.g. Gliwicz and Lampert, 1990) and/or digestibility (Van Donk et al., 1997; DeMott et al., 2010). Besides energy availability, zooplankton might be affected by the low nutritional quality of the food, because animals obtain a large set of essential or nearly essential requirements from their food (Sterner and Schulz, 1998). Many recent studies have investigated the performance of herbivores at imbalanced element to C ratios (Sterner and Elser, 2002) or imbalanced ratios of macronutrients (Raubenheimer and Simpson, 2004). Recent studies have focused on herbivore growth limitation by polyunsaturated fatty acids, amino acids, vitamins and sterols (Anderson et al., 2004; Wacker and Martin-Creuzburg, 2012). Sterols are essential food components for herbivorous arthropods (Behmer and Nes, 2003; von Elert et al., 2003), which cannot synthesize cholesterol (the predominant animal sterol) *de novo* but metabolize it from phytosterols in their diet (Svoboda and Thompson, 1985). Cholesterol serves as precursor for moult-inducing ecdysteroids in arthropods (Goad, 1981). Moreover, cholesterol is an indispensable component of plasma membranes and, because of its stabilizing properties (Robertson and Hazel, 1997), is necessary for membrane temperature adaptation (Crockett, 1998; Sperfeld and Wacker, 2009; Sperfeld and Wacker, 2011). Feeding on cyanobacteria can cause growth limitation of herbivorous crustaceans (von Elert et al., 2003) because cyanobacteria usually lack sterols (Volkman, 2003), an effect possibly exacerbated during cyanobacteria blooms (Wacker and Martin-Creuzburg, 2007). However, herbivore sterol limitation might not be restricted to cyanobacteria blooms as eukaryotic algae can also be poor in sterols. In particular, high light intensity and low nutrient availability (for instance in summer) can reduce sterol concentrations in algae below critical levels for herbivorous zooplankton such as *Daphnia* (Piepho et al., 2010; Piepho et al., 2012). Furthermore, not all phytosterols of eukaryotic algae are suitable precursors for cholesterol, and they vary in their conversion efficiency to cholesterol (Martin-Creuzburg and Von Elert, 2004). Thus, the growth of herbivorous crustaceans may depend not only on the amount of sterols in their diet but also on the phytosterol composition (Piepho et al., 2010), which is determined by the phytoplankton community composition.

Recent studies have focused mostly on the effects of different food conditions on herbivores' growth, but neglected measurements of C partitioning into different physiological fractions. However, such knowledge is very important to predict the contribution of herbivores to the overall C cycling in freshwater systems (He and Wang, 2006). *Daphnia* is a model organism of freshwater ecology for several reasons: for example, *Daphnia* plays an important ecological role as keystone species in aquatic ecosystems, there is a wealth of information about *Daphnia*'s biology, and its complete genomic information is available (Lampert, 2011). *Daphnia* has several behavioural and metabolic adaptations for dealing with food limitations. Low food quantity causes *Daphnia* to filter at a maximal rate to increase the C assimilation, and also results in an increase in the retention time of food in the gut (Geller, 1975; DeMott et al., 2010). Moreover, losses of C are diminished by reducing respiration (Lampert, 1986; Urabe and Watanabe, 1990; Schmoker and

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Hernandez-Leon, 2003). This response of the respiration rate to changing food concentrations refers to the increase in energy expenditure that occurs during digestion, and is defined as specific dynamic action (SDA) (e.g. Kiørboe et al., 1985; Lampert, 1986; Secor, 2009).

In contrast, when food quality is low, daphnids have to either improve the assimilation of the potentially limiting compound in their diet or get rid of the excess of other dietary ingredients, mostly C [see review by Hessen and Anderson (Hessen and Anderson, 2008) and model approach by Anderson et al. (Anderson et al., 2005)]. However, organisms may increase their fitness using this excess of C for other purposes, such as storage, structure and defence (Hessen and Anderson, 2008). Recent studies with phosphorus (P)-limited *Daphnia* showed adjustments in ingestion rate (Darchambeau and Thys, 2005) and assimilation efficiency of (excess) C and P (DeMott et al., 1998). Furthermore, daphnids may compensate for poor food quality by increasing C excretion (He and Wang, 2008) and respiration (Darchambeau et al., 2003; Jensen and Hessen, 2007), which depends on acclimation time (Lukas and Wacker, 2014). Unfortunately, food quality aspects based on the biochemical composition of the diet are missing in these studies. However, it is important to take the biochemical composition of food into account, because the incorporation of C into proteins, lipids and polysaccharides depends on it (Thor et al., 2002). Consequently, our purpose was to improve our knowledge of the processes that regulate C budgets for compounds such as sterols. Such information will be essential to understand the homeostatic regulation of sterols in daphnids (Sperfeld and Wacker, 2009), particularly because sterols (and other essential biochemical food components) contain C and are consequently part of the overall C pool of the animals. If *Daphnia* were not able to spare carbonic sterols from C losses by egestion of faeces, excretion and respiration, the elimination of excess C would be useless or even detrimental. Therefore, we hypothesize that *Daphnia* are able to selectively exclude sterols from C losses.

In this study, we acclimated *Daphnia magna* Straus 1820 to different regimes of food quantity and biochemical quality (cholesterol) and examined their C budgets by measuring egestion, excretion and respiration using the radiolabelled C method. Cholesterol budgeting was tested using radiolabelled cholesterol. We predicted that cholesterol would be spared from C losses (faeces egestion, excretion and respiration) when the animals fed on cholesterol-deficient diets.

RESULTS

Somatic growth

The growth of *D. magna* was limited by food quantity as well as food quality in terms of dietary cholesterol (two-way ANOVA, cholesterol: $F_{1,8}$=607.2, P<0.001, food quantity: $F_{1,8}$=510.7, P<0.001; Fig. 1). When the cholesterol concentration and food quantity was high (HC/HQ), *D. magna* reached the highest growth rate (P<0.001, Tukey's HSD). If either the quantity or the cholesterol content of food was low, growth rates of *D. magna* were reduced to the same moderate value (which apparently depends on the particular combination of cholesterol deficiency and food quantity limitation). At high food quantity (HQ), a low cholesterol concentration (LC) caused a strong decrease of *D. magna* growth rate by 50% compared with growth under HC/HQ. In contrast, when food quantity was low (LQ), a low food quality (LC) led to a decrease in growth of only 37% compared with growth under HC/LQ (ANOVA, two-way interaction, cholesterol × food quantity: $F_{1,8}$=111.5, P<0.001). When both food quantity and cholesterol concentration were reduced simultaneously (LC/LQ), the growth rate was lowest.

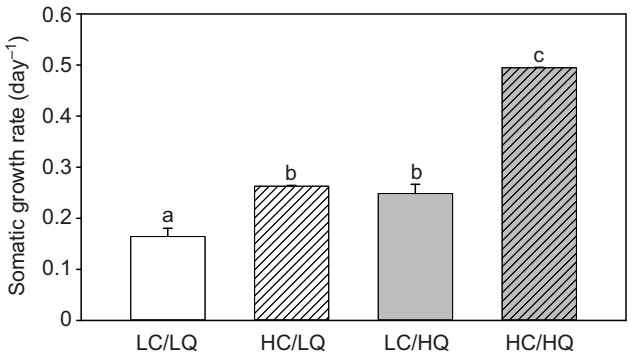

Fig. 1. Somatic growth rates of *Daphnia magna* fed with food of different quality and quantity. LC, low cholesterol; HC, high cholesterol; LQ, low food quantity; HQ, high food quantity. Data are means ± 1 s.d.; n=3. Statistically significant differences are indicated as different letters (Tukey's *post hoc* test, P<0.001).

Pulse-chase feeding experiment

In general, nearly all of the measured processes of *D. magna* C budgets were affected by the quantity of the food and its cholesterol concentration (two-way ANOVAs in Table 1). Excretion was the exception. Interestingly, neither food quantity nor food quality significantly affected C excretion in *D. magna*, although there was a marginal effect of cholesterol. The absolute values of measurements are displayed in supplementary material Table S1.

Assimilation efficiency, faeces and gross growth efficiency

Carbon assimilation efficiency (AE_C) was strongly affected by both food quantity and dietary cholesterol concentration (Table 1, Fig. 2). When the food quantity was low (LQ), AE_C was highest and did not differ between high (HC) and low cholesterol (LC) treatments (LC/LQ: 82.7±4.6%; HC/LQ: 88.6±3.5%, mean ± 1 s.d., n=5). However, at high food quantity (HQ), AE_C was generally lower and, additionally, it varied between high and low dietary cholesterol concentrations. Hence, we found the lowest AE_C when cholesterol was low and food quantity was high (LC/HQ: 50.7±3.1%; HC/HQ: 67.9±7.9%). This pattern of AE_C led to concordant results of faeces measurements and gross growth efficiency (GGE_C) calculations (Fig. 2). In agreement with the high AE_C at low food quantity, the egestion of faeces was low and did not differ between cholesterol concentrations. In contrast, at high food quantity, low dietary cholesterol resulted in a high egestion of faeces (Fig. 2). Hence, when *D. magna* were presented with high food quantity, GGE_C decreased at low cholesterol. In contrast, GGE_C did not differ between food qualities and was generally higher at low food quantity (Fig. 2).

Net growth efficiency, excretion and respiration

The C net growth efficiency (NGE_C), which contains information about the proportion of assimilated C used for production, showed a similar pattern as the GGE_C when food quantity and the cholesterol concentration in the food were changed (Fig. 3). At low food quantity, NGE_C was high and not different between the two cholesterol concentrations. At high food quantity, daphnids reached the same high NGE_C when cholesterol was non-limiting. In contrast to the results at low quantity, a lower cholesterol concentration led to a lower NGE_C when food quantity was high. In the latter scenario, we found an increased respiration at low dietary cholesterol and high food quantity (Fig. 3). At low food quantity, this effect of food quality on respiration was not present. The excretion rates of *D.*

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Table 1. Results of two-way ANOVA on the effect of food quality (cholesterol) and food quantity on several carbon pathways in *Daphnia magna*

	Cholesterol		Food quantity		Interaction	
Proportion (Efficiency)	$F_{1,16}$	P	$F_{1,16}$	P	$F_{1,16}$	P
Assimilation/ingestion (AE)	24.4	**<0.001**	130.8	**<0.001**	3.1	0.097
Faeces/ingestion	15.0	**0.001**	54.6	**<0.001**	4.6	**0.048**
Production/ingestion (GGE)	5.4	**0.033**	63.1	**<0.001**	12.5	**0.003**
Production/assimilation (NGE)	3.5	0.079	13.3	**0.002**	8.0	**0.012**
Excretion/assimilation*	3.7	0.073	2.2	0.16	0.8	0.39
Respiration/assimilation	7.9	**0.013**	33.1	**<0.001**	9.5	**0.007**

*d.f.=15.
AE, assimilation efficiency; GGE, gross growth efficiency; NGE, net growth efficiency.
Bold P-values indicate $P<0.05$.

magna were not significantly affected either by the food quantity or by the cholesterol concentration in the food (Table 1). Nevertheless, we found a marginal increase in excretion rate at low food quality (two-way ANOVA, $P=0.073$).

Cholesterol in C budgets

Compared with assimilation efficiencies (AE) of bulk C at high quantity (Fig. 2), the AE of cholesterol were high (ca. 86%) and did not differ between the two cholesterol concentrations (Fig. 4). Accordingly, the egestion of faeces was low at both concentrations, which resulted in high, non-varying gross growth efficiencies (production per ingestion) for cholesterol. Furthermore, the proportion of assimilated cholesterol used for production (net growth efficiency) was high, indicating respiration losses that were lower and even negligible compared with bulk C losses. The only significant effects of the dietary cholesterol concentration on the direct cholesterol metabolism were those on the excretion, which was higher at low dietary cholesterol compared with the non-limiting concentration (one-way-ANOVA, $F_{1,4}=3.2$, $P=0.022$; Fig. 4).

DISCUSSION

The present study revealed strong effects of sterol availability and food quantity on C assimilation and faeces egestion as well as respiration in *D. magna*. Moreover, we found that *D. magna* selectively exclude cholesterol from C losses such as faeces egestion and respiration. In the following we discuss the different C pathways in each of our four treatments and use the high cholesterol (HC)–high quantity (HQ) treatment as a reference.

High cholesterol–high quantity

In general, we produced evidence that effects of food quality strongly depend on food quantity, because *D. magna* growth reduction due to cholesterol limitation was diminished at low food quantity. The HC/HQ treatment had the highest growth rates of *D. magna*, which is consistent with recent results (Sperfeld and Wacker, 2009; Lukas et al., 2011). Moreover, the results for almost all measured C pathways of the present study (Fig. 5A) were similar to those from previous experiments with *Daphnia* grown under non-limiting food conditions (neither quantitatively nor qualitatively). Accordingly, we found comparable values for carbon assimilation efficiencies (AE_C), carbon gross growth efficiencies (GGE_C, production per ingestion) and carbon net growth efficiency (production per assimilation, NGE_C) (DeMott et al., 1998; He and Wang, 2008) as well as for respiration (Fedorov and Sorokin, 1967; He and Wang, 2006) and excretion of dissolved organic carbon (DOC) (Darchambeau et al., 2003; He and Wang, 2006). Only the results for the AE_C were somewhat contrasting, as we and DeMott et al. (DeMott et al., 1998) showed high values, but He and Wang (He and Wang, 2008) obtained much lower AE_C (indicating low and high faeces egestion, respectively). These differences in AE_C might be due to differences in food concentrations (DeMott et al., 2010), but the food concentration in our study and those from He and Wang (He and Wang, 2008) were more similar than those of DeMott et al. (DeMott et al., 1998). Moreover, AE_C might be age dependent, because animals from the He and Wang study (He and Wang, 2008) were much older (11 days) and had already transferred energy to their offspring with energy. In any case, we clearly show here that faeces egestion is a non-negligible fraction

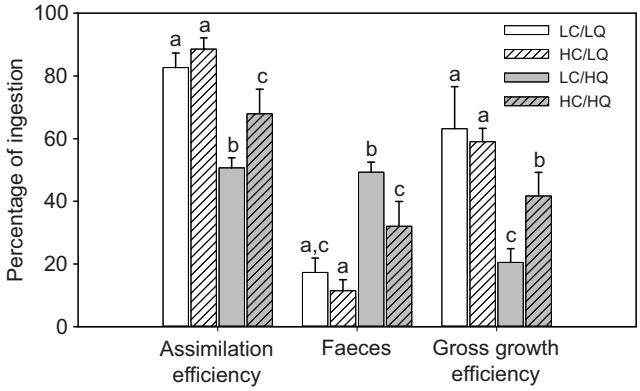

Fig. 2. Carbon assimilation efficiency (assimilation per ingestion), faeces egestion (per ingestion) and gross growth efficiency (production per ingestion) of *D. magna* with food of different quality and quantity. Data are means ± 1 s.d.; $n=5$. Statistically significant differences are indicated as different letters (Tukey's *post hoc* test, $P<0.05$).

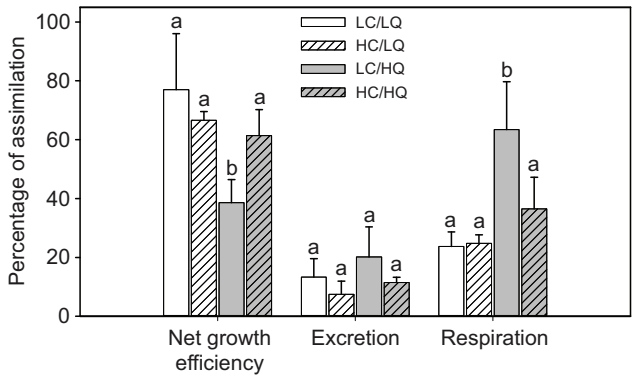

Fig. 3. Net growth efficiency (production per assimilation), excretion and respiration (both as proportions of assimilation) of *D. magna* with food of different quality and quantity. Data are means ± 1 s.d. with $n=5$, except for excretion at HC/LQ ($n=4$). Statistically significant differences are indicated as different letters (Tukey's *post hoc* test, $P<0.05$).

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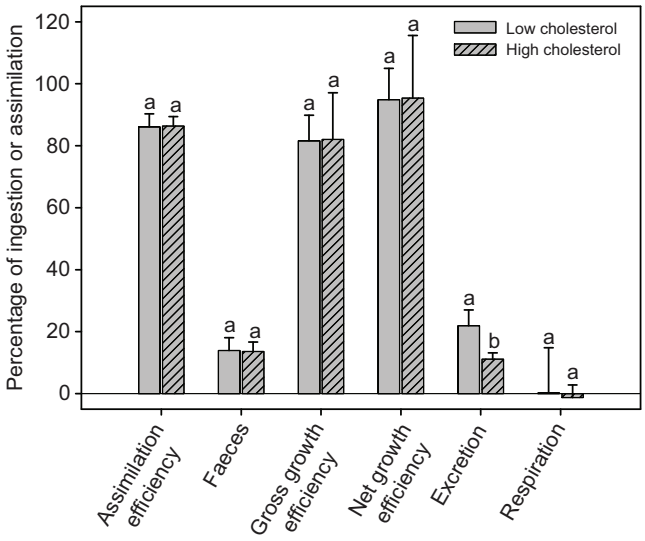

Fig. 4. Assimilation efficiency (assimilation per ingestion), egestion (faeces per ingestion), gross growth efficiency (production per ingestion), net growth efficiency (production per assimilation) and excretion and respiration (as proportions of assimilation) of cholesterol of *D. magna* acclimated (48 h) to two different cholesterol concentrations (low: 3.5 µg cholesterol mg^{-1} C, high: 14 µg cholesterol mg^{-1} C). Data are means ± 1 s.d.; *n*=3. Except for excretion (one-way ANOVA, $F_{2,6}$=13.2, *P*=0.022), all remaining one-way ANOVAs revealed no differences between low and high dietary cholesterol (*P*>0.85).

of C ingested – even under good food conditions. In contrast with green algae, the cyanobacteria *Synechococcus elongatus* we used does not have cell walls and is well digestible (Lampert, 1977). If *Daphnia* food supply consisted instead of green algae with strong cell walls (e.g. Van Donk et al., 1997) or gelatinous coverings that reduce digestibility (DeMott et al., 2010), faeces egestion might have been more pronounced. Unfortunately, it has not been well described, until now, how limitations of food quality (and quantity) affect *Daphnia*'s egestion of faeces (neither independently nor simultaneously). This knowledge gap shows how the defecation processes in daphnids have been neglected as an important part in the regulation of C budgets.

High cholesterol–low quantity

When we reduced the food quantity and kept food quality high (HC/LQ treatment), the growth of the animals decreased significantly to a moderate level. At low food quantity, the reduced ingestion was partly offset by increased AE_C (Fig. 5B); the higher AE_C probably derives from longer gut passage times at low food concentration (DeMott et al., 2010). Our results of very high AE_C at low food quantity corroborate earlier studies, which found higher AE_C at low compared with high food concentrations (Urabe and Watanabe, 1991; He and Wang, 2006). The faeces fraction was much smaller under food limitation and, therefore, it appears as a relatively minor route of C loss in energy-limited *Daphnia* (He and Wang, 2006). However, a modelling approach by Anderson et al. (Anderson et al., 2005) did not reveal lower faeces egestion at food limitation.

Interestingly, the GGE_C increased markedly when food concentration decreased, but the NGE_C did not change. Consequently, we suggest that in both low quantity treatments the efficiency from assimilation to production is very high. Our low food concentration was clearly limiting, but still provided enough energy for moderate growth. Therefore, our interpretation of the high values of GGE_C and NGE_C cannot be generalized to situations where food quantity approaches the threshold for zero growth. Then, the entire assimilated C is consumed as metabolic expenditure, and gross (and net) growth efficiency approaches zero (Lampert, 1977). Above such threshold concentrations, animals appear to respond differently to food limitation; we found still high NGE_C, which might originate from a very low excretion. As excretion of DOC did not change because of altered food quantity (see also He and Wang,

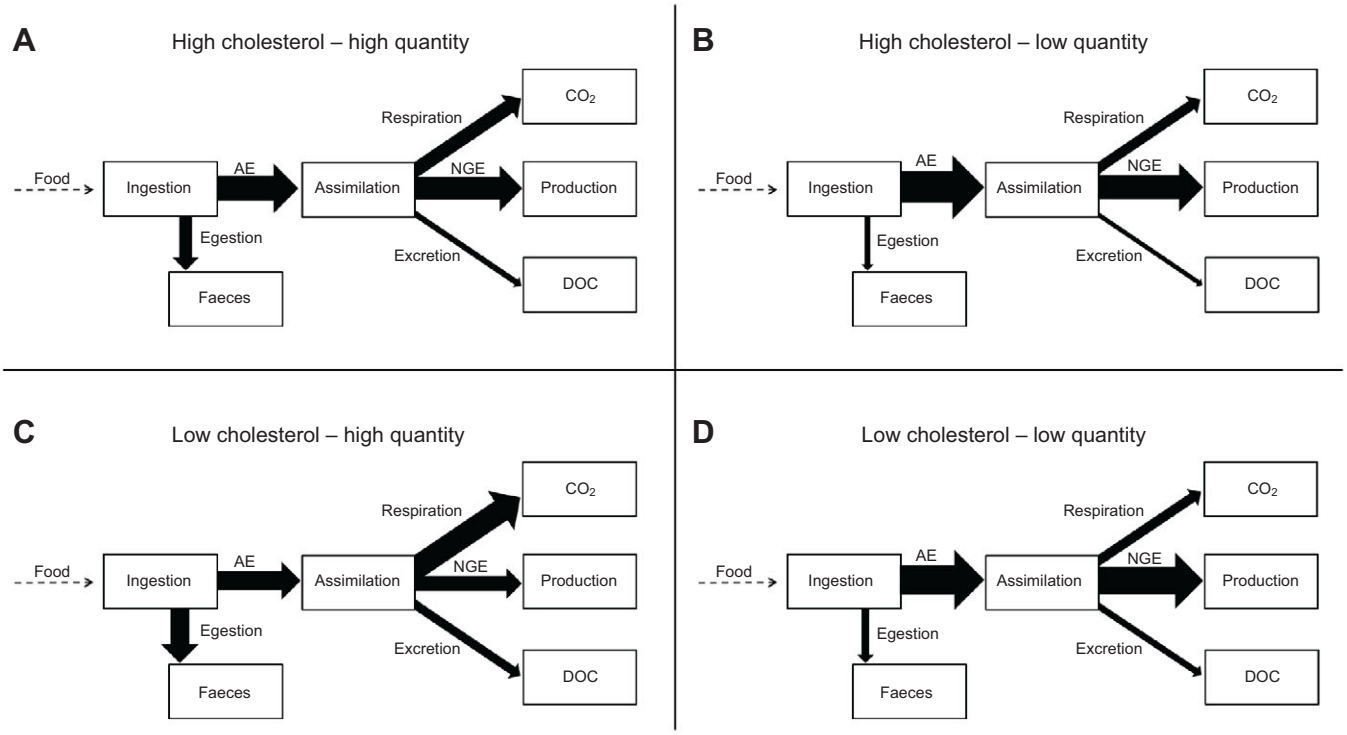

Fig. 5. Schematic of carbon pathways in *D. magna* grown under different food conditions. The thickness of the arrows indicates the relative value of the respective C pathways. AE, assimilation efficiency; DOC, dissolved organic carbon; NGE, net growth efficiency.

2006), we assume low respiration as another potential explanation for the high NGE_C observed in the HC/LQ treatment. In comparison with the HC/HQ treatment, HC/LQ respiration values were not significantly different, but, consistent with earlier studies, our results show a generally decreased respiration at lower food availability (food quantity effect in two-way ANOVA; Table 1) (Lampert, 1986; Schmoker and Hernandez-Leon, 2003; Anderson et al., 2005). This response of the respiratory rate to changing food conditions (termed SDA) refers to the increase in energy expenditure that occurs during meal digestion (e.g. Kiørboe et al., 1985; Secor, 2009).

Low cholesterol–high quantity

When C was available in excess relative to cholesterol (LC/HQ treatment), the growth rates of *D. magna* were reduced to the same moderate value as in the high cholesterol (HC)–low quantity (LQ) treatment. This indicates that ecologically relevant changes in food quantity and quality (in terms of cholesterol) can potentially have a similar impact on *Daphnia*, although this certainly depends on the particular combination of cholesterol deficiency and how much the food quantity is reduced below the incipient limiting level. The comparable responses of growth rates under food quantity or quality limitation, however, were not based on similar mechanisms. Instead they were the result of changes in C pathways.

In general, by using cholesterol as food quality indicator, the present study differs from previous studies examining the effect of P limitation on *Daphnia* C pathways. We conclude that *Daphnia* has a complex network of regulatory mechanisms for different types of limitations, i.e. we suggest that different strategies are used to handle limitations by cholesterol and elemental P. Interestingly, two important studies on C budgets in *Daphnia*, when dietary P supply was changed, differed in their results (DeMott et al., 1998; He and Wang, 2008) and make comparisons difficult. Because we did not address P supply, in the following we focus on the biochemical scope of our study.

We found the lowest AE_C at low food quality and high quantity (Fig. 5C), which confirmed the results of DeMott and Müller-Navarra (DeMott and Müller-Navarra, 1997), who found reduced AE_C for *Daphnia* feeding on the cyanobacterium *S. elongatus* alone, but higher AE_C when *S. elongatus* was provided in combination with green algae of high food quality. Low AE_C in the present study led to high egestion of faeces, stressing the importance of faeces egestion in *Daphnia* C budgets, i.e. *Daphnia* uses faeces as a highly effective means to get rid of excess C when food quality, in terms of cholesterol, is limiting. In correspondence with low AE_C and high faeces egestion, we found the lowest GGE_C at high food quantity but low cholesterol availability. Low GGE_C and high faeces egestion at low food quality were supported by DeMott et al. (DeMott et al., 1998), but not by He and Wang (He and Wang, 2008). The latter showed longer gut passage times for P-limited animals, a fact that suggests lower faeces egestion.

We also found the lowest NGE_C at limiting cholesterol concentrations compared with our other treatments. Low NGE_C could be the result of high DOC excretion, which can be the predominant component of C release under P-limited conditions (Darchambeau et al., 2003; Anderson et al., 2005; He and Wang, 2008). However, in contrast to results for P-deficient diets, we did not find significant effects of cholesterol on the excretion of DOC. This clearly indicates that *Daphnia* responds differently to biochemical limitation such as cholesterol, compared with P limitation. At any rate, our data suggest a marginal increase in excretion rate as a mechanism to get rid of excess C at low food quality in terms of biochemicals, e.g. sterols (two-way ANOVA, effect of cholesterol on DOC excretion: P=0.073). Nevertheless, care should be taken when interpreting excretion data, as one has to distinguish between direct excretion of DOC and the release of DOC from faecal material. He and Wang (He and Wang, 2006) suggested that faeces leakage was only a small fraction of total DOC release. Our results are consistent with this, because we did not find a correlation between the proportions of faeces and excretion (linear regression, R^2=0.14, P=0.12). Hence we assume leakage of faeces into the DOC pool was negligible. A further reason for *Daphnia*'s low NGE_C in our study is certainly the strong increase in respiration when cholesterol was low, as previously shown for *Daphnia* fed with P-limited diets (Darchambeau et al., 2003; Jensen and Hessen, 2007). Nevertheless, the results of Jensen and Hessen (Jensen and Hessen, 2007) are not directly comparable with ours and those of Darchambeau et al. (Darchambeau et al., 2003). The differences may stem from different methods of determination of respiration rates, i.e. consumption of O_2 (Jensen and Hessen, 2007) versus release of $^{14}CO_2$ (Darchambeau et al., 2003; present study). The respiratory quotient (defined as the volume of CO_2 produced per volume of O_2 consumed) will be affected by the biochemical make-up of the algal food (Jensen and Hessen, 2007).

Low cholesterol–low quantity

When *Daphnia* was grown in the worst food treatment (low quantity and quality, LC/LQ) the patterns in C pathways were not different from those of the HC/LQ treatment. Accordingly, *Daphnia* C pathways are not affected by food quality (in terms of cholesterol) as long as food quantity is also limiting. As C and cholesterol assimilation efficiencies were high in both low quantity treatments (LC/LQ and HC/LQ), the animals grew better at higher cholesterol availability (HC/LQ) than at lower cholesterol availability (LC/LQ).

The C losses of *Daphnia* are reduced by decreasing all C-costly processes such as excretion, respiration and faeces egestion (Fig. 5D). Less egestion of faeces is probably the result of low ingestion rate and slow gut passage at low food concentration. To our knowledge, the present study is the first to show concurrent effects of low food quantity and quality on *Daphnia* and how animals adjust C budgets and regulate growth in response to it. We illustrate the dilemma of animals of adjusting C budgets for low quality at low quantity. When food quantity was high, *Daphnia* was able to adjust C pathways for differences in dietary cholesterol (e.g. by higher faeces egestion or respiration), but the constraints imposed by low food quantity superseded other possible adjustments due to low food quality.

Pathways of essential organic compounds

Until now, discussions on the regulation of zooplankton C pathways have focused on bulk C, but here we start investigating the pathways of essential biochemical compounds. The pathways of bulk C differ significantly from those of cholesterol. We found AE_C to be lowest at low cholesterol concentrations, but the assimilation efficiencies of cholesterol itself were much higher. Thus, our results show a strong retention of this essential food compound which increases our knowledge about the reaction of *Daphnia* to low biochemical food quality. Comparable to our findings, recent results describe lower AE_C but higher assimilation efficiencies for P in P-limited *Daphnia* (e.g. DeMott et al., 1998). Only when we set cholesterol concentration to very high values (above 55 µg cholesterol mg^{-1} C), probably not found in nature, did the AE of cholesterol decrease and the excretion of cholesterol increase (60% and 30%, respectively). This response explains the accumulation and loss of cholesterol at low versus high cholesterol levels, respectively, and enables *Daphnia* to maintain a suitable cholesterol concentration in the tissues (within ecologically relevant scales of ca. 5–10 µg cholesterol mg^{-1} C) (Sperfeld and Wacker, 2009).

The egestion of bulk faeces increased when the cholesterol concentration in the food was low, but the egestion of cholesterol via faeces was very low. Accordingly, *Daphnia* achieved strong regulation (after ingestion) of this essential biochemical by improving low cholesterol:C ratios: (1) *Daphnia* increased egestion of excess C and (2) simultaneously retained cholesterol from egestion. Because of low egestion and selective retention of cholesterol, ~80% of the ingested cholesterol was used for production. A similar value was found for the GGE of P when *Daphnia* was fed P-limited algae (89%, DeMott et al., 1998). Interestingly, our NGEs for cholesterol were consistently high, while DeMott et al. (DeMott et al., 1998) found decreasing phosphorus NGEs for P-limited *Daphnia*. They explained this by a low, but consistent, P excretion even in strongly P-limited *Daphnia*. Similarly, we found measurable cholesterol excretion even though cholesterol was limiting. Hence, the mechanism of higher DOC excretion to get rid of excess C at low cholesterol concentrations (as indicated in our study) seems to be inoperative. Consequently, excretion of C derived from essential carbonic compounds is not independent from bulk C excretion, which is clearly in contrast to non-carbonic compounds such as minerals (Frost et al., 2004; He and Wang, 2008). As an explanation, we suggest that *Daphnia* does not distinguish between essential and non-essential C compounds in the excretion pathway: a higher excretion of excess C at low food quality simultaneously causes higher excretion of carbonic cholesterol.

In contrast, although the isotope method we used provides a very sensitive measure, we did not observe a detectable respiration of cholesterol; therefore, cholesterol respiration is very low and not significantly different from zero. Hence, in addition to the faeces regulation, we found two further mechanisms by which *Daphnia* can improve low cholesterol:C ratios – they increase respiration of excess C, while sparing cholesterol from respiration at the same time. With this conclusion, we emphasize that many biochemical food components contain C and are consequently part of the overall C pool of the animals. Especially when these components are only a small part of the overall C content, *Daphnia* should handle such carbonic essential molecules efficiently. This fact needs to be considered for analyses of C budgets, when essential food compounds that contain C are investigated. Additionally, co-limiting scenarios (e.g. co-limitation by cholesterol and P or by cholesterol and polyunsaturated fatty acids) lead to interactions between co-limiting nutrients (Lukas et al., 2011; Sperfeld et al., 2012) and consequently also C budgeting of co-limiting carbonic nutrients may interact. Such interactive effects on C budgeting of animals *in situ* might be identified by nutritional indicators (Wagner et al., 2013).

In conclusion, our results clearly indicate that *Daphnia* varies its regulation of C losses in response to different food conditions. In particular, the effects of food quality in terms of cholesterol are important in several C pathways, given that food quantity was non-limiting. This provides further evidence of stronger effects of food quality on zooplankton at non-limiting food quantities, which were previously described for growth only (see Sterner and Schulz, 1998). Moreover, increased discharge of bulk C and simultaneous high retention of cholesterol imply that *Daphnia* is able to adjust C budgets and achieve moderate growth rates at low cholesterol availability.

MATERIALS AND METHODS

Organisms

The stock culture of *D. magna* was grown in filtered water (0.2 µm pore-sized membrane filter) from Lake Stechlin (northeast Germany) with 2 mg C l^{-1} of the green algae *Scenedesmus obliquus* (SAG 276-3a, culture collection Goettingen, Germany) as food. For the growth experiment, the easily ingestible, non-toxic and P-saturated cyanobacterium *Synechococcus elongatus* was used as food for *D. magna*. *Synechococcus elongatus* (SYN; SAG 89.79), lacking sterols and polyunsaturated fatty acids (von Elert et al., 2003), was cultured in aerated 2-l flasks containing Wright's cryptophyte (WC) medium with vitamins (Guillard, 1975) and diluted daily (dilution rate 0.2 day^{-1}) in order to ensure nutrient repletion. The culture was maintained at an illumination of 40 µmol photons m^{-2} s^{-1} using a 16 h:8 h light:dark cycle. All organisms were raised at 20°C.

Experimental design and procedure

In order to examine the simultaneous dependency of *D. magna*'s growth and C budgets on food quality and quantity, we supplied *Daphnia* with two different dietary concentrations of cholesterol and two food concentrations in a full-factorial design (Table 2). The 'high quality' treatments provided enough cholesterol so that it was not limiting (Sperfeld and Wacker, 2009), but was not a substantial excess. We used liposomes loaded with cholesterol to control food quality (see Liposome preparation, below). Third-clutch juveniles used for the experiment were collected within 12 h from mothers that were transferred to jars with cholesterol-deficient food (SYN, 2 mg C l^{-1}) in the beginning of the 12 h. By doing so, we avoided a temporally different cholesterol supply to newly hatched juveniles, and thus a potentially confounding variation in cholesterol storage as previously shown for P (Lukas et al., 2013). A subset of these juveniles was dried and weighed for the determination of the initial dry mass.

The treatments with food quantity and/or quality limitation each started with 320 neonates that were randomly distributed into eight replicate jars. For the treatment without any limitation, 160 neonates were used. In order to consider the different sizes of the daphnids and to avoid a depletion of food, the volumes of food suspension were adjusted as follows: animals with high food concentration were raised in jars containing between 30 ml per individual at the beginning and 60 ml per individual at the end of the experiment. Animals with low food concentration were raised in jars containing between 80 ml per individual at the beginning and 200 ml per individual at the end of the experiment. In order to do so, we used up to five 2000 ml jars for one replicate. Throughout the experiment, daphnids were transferred daily into jars with renewed food suspensions. The growth experiment was terminated after 5 or 6 days for high food quantity (HQ) and low food quantity (LQ), respectively, in order to allow animals with LQ to reach a size comparable to animals with HQ. The daphnids of each treatment were split into two groups. One group (five replicates) was used for the pulse-chase feeding experiment (see below). The remaining daphnids (three replicates) were rinsed with ultrapure water and transferred into pre-weighed aluminium boats. After drying for 48 h at 50°C, daphnids were weighed on an electronic balance (±1 µg; CP2P, Sartorius, Goettingen, Germany). The somatic growth rates (g) were calculated as the change in dry mass per individual from the beginning ($M_{dry,0}$) to the end of the experiment ($M_{dry,t}$) using the equation:

$$g = [\ln(M_{dry,t}) - \ln(M_{dry,0})] \times t^{-1}, \quad (1)$$

where t is the duration of the experiment in days.

Table 2. Conditions used during the growth and pulse-chase experiment

Treatment	Cholesterol (µg mg^{-1} C)	Food quantity (mg C l^{-1})
Low cholesterol–low quantity (LC/LQ)	3.5	0.2
Low cholesterol–high quantity (LC/HQ)	3.5	2
High cholesterol–low quantity (HC/LQ)	17.5	0.2
High cholesterol–high quantity (HC/HQ)	17.5	2

We used a cross-way scheme of concentrations of dietary cholesterol and food quantity.

Liposome preparation

Cholesterol containing liposomes and empty liposomes without further ingredients were prepared according to Wacker and Martin-Creuzburg (Wacker and Martin-Creuzburg, 2012). Cholesterol liposomes were used as food supplements in growth experiments and during the pulse-chase feeding experiment. The overall C content of the liposome solution (liposomes plus cholesterol in buffer) was 2 mg C ml^{-1}. Accordingly to the supplemented volumes of liposomes (low cholesterol: 10 µl liposomes mg^{-1} C, high cholesterol: 50 µl liposomes mg^{-1} C), the C concentrations of the low quality treatments increased by 2%, and those of the high quality treatments by 10% (i.e. the C increase from low to high quality accounted for 8% in each food quantity). This additional C could be considered negligible when compared with the increase by 900% when food quantity was changed from low to high concentrations. The amount of cholesterol in subsamples of liposomes was determined using gas chromatography according to Martin-Creuzburg et al. (Martin-Creuzburg et al., 2009). For the calculation of C-based cholesterol concentrations, the amount of cholesterol added by liposomes was related to the POC concentrations of *S. elongatus* in food suspensions. To obtain radiolabelled cholesterol liposomes we loaded empty liposomes with radiolabelled (^{14}C) cholesterol (American Radiolabeled Chemicals Inc., St Louis, MO, USA); empty liposomes were sonicated for 15 min, followed by 1 h incubation with ^{14}C-cholesterol (50 mCi $mmol^{-1}$). To this we added ^{14}C-cholesterol in the same concentration as that used for the non-radiolabelled cholesterol liposomes (333 µg ml^{-1}). To verify the efficient incorporation of ^{14}C-cholesterol into the liposomes, we filtered the reassembled liposomes on Nucleopore filters (0.2 µm, 25 mm, Whatman International Ltd, Maidstone, UK). Less than 5% of the initial ^{14}C-cholesterol concentration was detected in the filtrate and more than 95% in the reassembled liposomes on the filter.

Pulse-chase feeding experiment

In order to investigate the C budgets of *D. magna*, we used the radiotracer technique (radioactively labelled C; ^{14}C) to follow the allocation of C into different compartments including respiration of dissolved inorganic carbon (DIC), excretion of dissolved organic carbon (DOC) and egestion of particulate organic carbon (POC) as faeces.

Exponentially growing SYN was labelled with ^{14}C from $NaH^{14}CO_3$ (1 mCi l^{-1}) until cells were uniformly radiolabelled after 4 days (specific radioactivity of 2.3–2.6×10^7 dpm mg C^{-1}). Before using them as diet, the food suspension was supplemented with liposomes containing cholesterol according to the experimental protocol (Table 2).

For the pulse-chase feeding experiment, *D. magna* was previously acclimated on non-radiolabelled experimental diets (see Experimental design and procedure, above) and then exposed to the radiolabelled diets (five times replicated) for 5 min (pulse). This is expected to be much shorter than the gut passage time of approximately 10–15 min and avoids the defecation of radiolabelled faeces (He and Wang, 2008). After such pulse feeding, the animals were rinsed with radioactive-free medium and transferred into non-radiolabelled experimental food suspensions (chase) under dim light using completely filled 5 ml snap cap vials. During pulse and chase phases, food suspensions had the same quantity and quality characteristics as during the growth experiment. To avoid recycling of ^{14}C (e.g. re-uptake by daphnids), daphnids were rinsed with radioactive-free medium to avoid a carry-over of ^{14}C and were transferred into vials with new food suspension in regular time intervals (after 0.5, 1, 2, 4 and 6 h). Using a preliminary test (see supplementary material Fig. S1) we concluded that *Daphnia* stopped the incorporation of ^{14}C into the somatic tissue after 6 h, for which reason we confined our measurements to this time. To measure the amount of egested, excreted and respired ^{14}C during each time interval, subsamples of the food suspension including faeces (total fraction) were taken immediately after each time interval and the activity was instantly determined via liquid scintillation counting (0.5 ml sample + 2.5 ml Hionic Fluor scintillation fluid in liquid scintillation counter Tri-Carb 2810Tr, both PerkinElmer, Rodgau, Germany). The external standard ratio method was used for quenching and conversion from counts per minute (cpm) to disintegrations per minute (dpm) was corrected. By different fractionation we gained information about the amount of faeces egested (POC, $PO^{14}C$), respired $^{14}CO_2$ and excreted DOC ($DO^{14}C$). Therefore, the radioactivity in the total fraction ($DI^{14}C$+$DO^{14}C$+$PO^{14}C$) was measured directly, and after addition of hydrochloric acid (100 µl of 1 mol l^{-1} HCl in 4 ml of the sample) plus bubbling with air. By adding HCl, DIC ($DI^{14}C$) was all dehydrated to $^{14}CO_2$, which out-gassed; DOC ($DO^{14}C$) and POC ($PO^{14}C$) remained in the solution ($DO^{14}C$+$PO^{14}C$-fraction). Radioactivity of this fraction was measured. Subsamples of the $DO^{14}C$+$PO^{14}C$ fraction were membrane filtered (2 ml), and the retained particles on membrane filter were transferred into scintillation vials. After the filter was dissolved in 0.5 ml Soluene 350 (PerkinElmer), the radioactivity of POC ($PO^{14}C$) was determined. This particulate fraction was used as a measure for the faeces, because algal C in chase suspensions was not labelled with ^{14}C. We ensured that bubbling the total fraction with air had no influence on the amount of measured faeces, as we found no differences between the aerated und unaerated $PO^{14}C$ fractions (two-sample *t*-test, t=1.03, d.f.=198, P=0.31). By using the difference between the total fraction ($DI^{14}C$+$DO^{14}C$+$PO^{14}C$) and the $DO^{14}C$+$PO^{14}C$ fraction, the $DI^{14}C$ (=respiration) was calculated. The $DO^{14}C$ (=excretion) was calculated as the difference between the $DO^{14}C$+$PO^{14}C$ fraction and the $PO^{14}C$ fraction.

Immediately after the pulse-feeding (5 min) and after the last time interval (6 h), subsamples of the daphnids were taken: 10 animals each for the treatment without any limitation and 20 animals for each treatment with food quantity and/or quality limitation. Animals were then instantly digested in solubilizer (0.5 ml Soluene 350) and their radioactivity was determined via liquid scintillation counting. The measurement of animals after the pulse-feeding was used as value for ingested ^{14}C. After 6 h, we assumed that the measured ^{14}C in the animals was used for biomass production. We calculated the amount of C in each fraction by dividing the amount of measured ^{14}C by the ratio between ^{14}C and bulk C that was found in the radiolabelled diet. Resulting values were related to the C content of the animals, which was calculated using the determined dry mass of an unlabelled subsample of the daphnids and a previously determined conversion factor of 0.41 µg C $µg^{-1}$ dry mass.

Cholesterol in C budgets

To follow the fate of the biochemical in the measured C pathways and test the hypothesis of cholesterol exclusion from C budgeting, we ran a separate experiment and used radiolabelled cholesterol (in liposomes, see Liposome preparation, above) and the sterol-free cyanobacteria SYN. Using eukaryotic diets instead (e.g. green algae) would be problematic as these contain phytosterols that are radiolabelled by incorporating bulk ^{14}C derived from $NaH^{14}CO_3$ incubation. Consequently, the ^{14}C signal of phytosterols and of other C compounds in the different C fractions of *Daphnia* would not have been separated from each other. Juvenile daphnids (three replicates, 32 animals each) were acclimated on two different cholesterol concentrations (low: 3.5 µg cholesterol mg^{-1} C, high: 14 µg cholesterol mg^{-1} C) for 48 h and then used for a pulse-chase feeding experiment as above, except that radiolabelled cholesterol (in liposomes) was used during the pulse part instead of bulk ^{14}C. We assumed a homogeneous distribution of the radiolabelled liposomes in the prepared food suspensions and, accordingly, assumed *Daphnia*'s food to be uniformly labelled. The ^{14}C measured afterwards in each fraction was directly derived from ^{14}C-cholesterol. Furthermore, we assumed that the acclimation periods to sterol-limited conditions (48 h for the experiment with radiolabelled cholesterol as well as 5–6 days for the experiment with radiolabelled bulk C) are appropriate time scales for detecting differences in C pathways due to cholesterol limitation, though different times of acclimation to the limitation may have influenced the experimental outcome (Lukas and Wacker, 2014).

Statistical analysis

The dependency of *Daphnia*'s growth rate on high/low food quantity as well as high/low cholesterol was analysed using a full-factorial two-way ANOVA. We also analysed the influence of the different food conditions on the C budgets of the animals. Therefore, we calculated the proportions of assimilation (=ingestion–faeces), faeces and production of ingestion and the proportions of production, excretion and respiration of assimilation. We defined carbon assimilation efficiency (AE_C)=assimilation/ingestion, carbon gross growth efficiency (GGE_C)=production/ingestion and carbon net growth efficiency (NGE_C)=production/assimilation. For the ANOVA, proportions were transformed by arcsine-square root and the significance of differences among means was tested using multiple comparisons (*post hoc*

Tukey's test). All statistical analyses were carried out using the statistical software package R version 2.5.1 (R Development Core Team, 2007).

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Competing interests
The authors declare no competing financial interests.

Author contributions
Both authors contributed significantly to the conception, design and execution of the study, interpretation of the findings as well as drafting the article.

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