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Habitat temperature is an important determinant of cholesterol contents in copepods

R. Patrick Hassett[1,2,*] and Elizabeth L. Crockett[1,2]

[1]Department of Biological Sciences, Ohio University, Athens, OH 45701, USA and [2]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672, USA

*Author for correspondence (e-mail: hassett@ohiou.edu)

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SUMMARY

Effects of habitat and acclimation temperature on cholesterol contents were examined in oceanic and inshore species of copepods. The cholesterol content of five species of thermally acclimated copepods was determined, and nine species (representing six families) were sampled to assess the role of habitat temperature. The species selected have maximum habitat temperatures (and temperature tolerances) that vary at least twofold. Levels of dietary cholesterol required to achieve maximum growth were also studied at different acclimation temperatures in a eurythermal copepod. Both eggs and copepodites of *Calanus finmarchicus* had higher cholesterol levels at the warm acclimation temperature (16°C) than at the cooler temperature (6°C). Neither *Acartia tonsa*, *Acartia hudsonica*, *Temora longicornis* nor *Eurytemora affinis* altered cholesterol contents with acclimation temperature. Maximum growth rates were achieved at fourfold higher concentrations of dietary cholesterol in warm-acclimated *Eurytemora affinis* than in cold-acclimated animals. The most consistent trend is the positive relationship between cholesterol content and habitat temperature. Species residing in warmer habitats (e.g. *Centropages typicus*, *Eurytemora affinis*) had approximately twice the cholesterol of species living in colder waters (e.g. *Calanus glacialis*, *Euchaeta norvegica*). A similar pattern was observed for comparisons of species within genera (*Calanus*, *Acartia* and *Centropages*), with the species abundant at lower latitudes having more cholesterol than the northern congener. These data indicate that habitat temperature is an important determinant of cholesterol content, and cholesterol endows membranes with the stability required for a range of body temperatures.

Key words: cholesterol, copepod, zooplankton, temperature adaptation, temperature acclimation.

INTRODUCTION

Cholesterol is the most abundant neutral lipid in the cell membranes of animals. It stabilizes membrane structure (Bloom and Mouritsen, 1988), limits membrane permeability (Kroes and Ostwald, 1971) and imparts a suitable working environment for membrane proteins (Yeagle et al., 1988). Despite the physicochemical influences of cholesterol, its distribution in biological membranes is far from homogenous. Cholesterol is primarily localized to the plasma membrane (Lange et al., 1989). Even within the plasma membrane cholesterol associates with sphingolipids and can concentrate in microdomains, including membrane rafts and caveolae, providing a platform to organize glycosylphosphatidylinositol-anchored proteins (Sengupta et al., 2007).

Because of its integral roles in biological membranes, cholesterol is considered a key constituent for animal growth, and requirements for cholesterol are likely to be driven largely by its contents in biological membranes. Crustaceans, like many other invertebrates, are considered unable to synthesize cholesterol *de novo* (Goad, 1981) and therefore must rely on exogenous sources of sterol for somatic and reproductive growth. This inability to synthesize cholesterol may be particularly challenging for many species of copepods since the sterol content and composition of their phytoplankton diet can vary greatly (e.g. Ballantine et al., 1979; Patterson et al., 1993; Patterson et al., 1994; Barrett et al., 1995; Véron et al., 1996). Cholesterol has been shown, in fact, to be a limiting factor in the growth of several crustaceans (Von Elert et al., 2003; Martin-Creuzburg and Von Elert, 2004; Hassett, 2004). Recently, we have shown that both egg production and viability are enhanced in copepods fed a diatom diet supplemented with cholesterol, while at the same time cholesterol contents of biological membranes are unaffected (Hassett, 2004; Crockett and Hassett, 2005). These results demonstrate that assimilated cholesterol (beyond what is present in the phytoplankton diet) is utilized for reproductive growth and that cholesterol levels in membranes are regulated, presumably in order to preserve membrane physical properties and function.

Cholesterol contents of organisms that live at different body temperature may require fine-tuning in order to stabilize physical properties of membranes (Hazel and Williams, 1990; Crockett, 1998). Like low temperature, cholesterol orders fluid-phase membranes, leading one to predict that cholesterol levels rise with temperature in order to counter the fluidizing influence of elevated temperatures. Temperature has been shown to affect membrane lipid composition and physical properties in a variety of crustaceans including crayfish (Pruitt, 1988), amphipods (Lahdes et al., 2000), crabs (Cuculescu et al., 1995) and copepods (Farkas et al., 1988), yet relatively little work has examined more specifically how cholesterol levels in crustaceans may be affected by temperature. Since copepods are among the most abundant animals on earth, and play a central role in marine foodwebs (Mauchline, 1998), it is important to understand how temperature may influence the copepod's requirements for cholesterol.

To determine whether and how cholesterol contents vary with temperature in copepods, we surveyed a range of species within the crustacean subclass Copepoda (order Calanoida). For temperature acclimations, several oceanic and inshore species were used. We examined further the relationship between cholesterol content and habitat temperature by comparing the cholesterol content in nine species of copepods, representing six families, whose maximum

Table 1. Phylogenetic relationships among 10 species of marine copepods (class Crustacea) used in this study

Subclass	Order	Superfamily	Family	Secies
Copepoda	Calanoida	Centropagidea	Acartidae	*Acartia tonsa*, *Acartia hudsonica*
			Centropagidae	*Centropages typicus*, *Centropages hamatus*
			Temoridae	*Temora longicornis*, *Eurytemora affinis*
			Tortanidae	*Tortanus discaudatus*
		Megacalanoidea	Calanidae	*Calanus finmarchicus*, *Calanus glacialis*
		Clausocalanoidea	Euchaetidae	*Paraeuchaeta norvegica*

Classification based on Mauchline (1998).

habitat temperatures and temperature tolerances span a range of at least twofold. We also evaluated the influence of acclimation temperature on the level of dietary cholesterol necessary to achieve maximum growth.

MATERIALS AND METHODS

Animal collections and acclimations

Eurytemora affinis Poppe was collected from an estuary (Northeast Creek) on Mount Desert Island, ME, USA with a 202 μm mesh dip net. *Acartia tonsa* Dana, *A. hudsonica* Pinhey, *Temora longicornis* Müller, *Tortanus discaudatus* Thompson and Scott, and *Centropages hamatus* Lilljeborg were collected from Frenchman Bay, adjacent to Mount Desert Island, with a 0.5 m 202 μm mesh net, 0–5 m horizontal tow. *Calanus finmarchicus* Gunnerus and *Calanus glacialis* Jaschnov were collected by the RV *Indigo* (College of the Atlantic) 5 mi offshore of Mount Desert Island in the Gulf of Maine with a 350 μm 0–50 m vertical tow. Additional samples for cholesterol content of *Paraeuchaeta norvegica* Boeck and *Centropages typicus* Kröyer were taken from frozen samples (stored over liquid nitrogen) that had been previously collected with a MOCNESS net during the US GLOBEC RV Endeavor cruise 330 in the central Gulf of Maine, October 1999. Phylogenetic relationships among the species of calanoid copepods are presented in Table 1.

Animals used in acclimations were held in one-liter containers for 7–10 days at high (16–25°C) and low (6°C) temperatures in two refrigerators equipped with BOD-cubator temperature controls (N-Con Systems, Crawford, GA, USA). Temperatures were selected to reflect the temperature range of the species in the field, and so were not identical for each species. In addition to acclimation at cold temperature (6°C), *Aeartia hudsonica* and *Temora longicornis* were acclimated at 18°C, the more warm-tolerant *A. tonsa* and *Eurytemora affinis* at 22°C and 25°C, respectively. *C. finmarchicus* CVs (fifth copepodite stage) were acclimated at 6°C and 16°C, whereas eggs were collected from females acclimated at 6°C and 12°C since egg production was very low at 16°C. Bradley (Bradley, 1978) found that *E. affinis* acclimated measurably in 3 h, and completely within 2–4 days, so the 7–10 days acclimation period should allow sufficient time to achieve full acclimation. Experiments with *C. finmarchicus* were conducted mid-to-late June, *T. longicornis* and *A. hudsonica* in early July, *A. tonsa* in late July, all in 2005, and *E. affinis* in early July 2007. These time periods correspond to periods of high abundance of these species in the plankton. Animals were fed daily with stabilized, concentrated *Tetraselmis–Nannochloropsis* cultures (Reed Mariculture, Campbell, CA, USA) and water was changed every other day. Although the sterol composition of the *Tetraselmis–Nannochloropsis* diet is not known with certainty, cholesterol is the dominant sterol in *Nannochloropsis* (Patterson et al., 1994), and is abundant, and sometimes dominant, in different strains of *Tetraselmis* (Patterson et al., 1993). Other sterols are dominated by 24-methylenecholesterol and 24-methylcholesterol (*Tetraselmis*) and 24-ethylcholesterol (*Nannochloropsis*), which are Δ^5 sterols that may be de-alkylated to cholesterol (Prahl et al., 1984). At the end of the acclimations, animals were concentrated, sorted for dead or moribund individuals, separated on a 60 μm Nitex screen, and frozen over liquid nitrogen for later analysis of cholesterol and protein content.

For the growth rate experiment *Eurytemora affinis* females and males were held in 20 liter carboys and fed for 5 days on the *Tetraselmis–Nannochloropsis* culture. Adults were then concentrated and transferred to a clean carboy with filtered seawater. Animals were held overnight and newly hatched nauplii were separated and raised in 250 ml containers in two small, Peltier-cooled incubators (Incufridge; Revolutionary Science Inc, Lindstrom, MN, USA) at 6°C and 25°C at 20 p.p.t. salinity, with one incubator for each temperature. Animals for the growth experiment were fed the cyanobacterium *Synechococcus bacillus* (CCMP 1261, cell dimensions of 4–8 μm×2 μm) supplemented with cholesterol over a range of concentrations. Cultures were obtained from the Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, Boothbay Harbor, MA, USA) and maintained on K medium (Keller et al., 1987) at 20°C. *S. bacillus* was added at approximately 500 μg cholesterol l^{-1}, estimated from literature values of cholesterol per cell (Liu et al., 1999). This food level is in excess of saturating levels of 300 μg cholesterol l^{-1} (Barthel, 1983). The *S. bacillus* culture was supplemented at 0.01, 0.05, 0.1, 0.25 and 0.5 μg cholesterol l^{-1} (=0.005–0.25% cholesterol by weight), with two replicates at each concentration. Preliminary experiments indicated that no growth occurred on a *S. bacillus* diet in the absence of supplementation, so a pure cyanobacterium diet was not used in the subsequent experiment. In addition, the preliminary experiment indicated that growth rates reached a maximum at lower cholesterol concentrations at 6°C, so a treatment at 0.025 μg cholesterol l^{-1} was substituted for the 0.25 μg l^{-1} in the cold treatment. The use of a cyanobacterium (with its characteristic absence of cholesterol) allows control of the cholesterol content without the confounding factor of temperature, which can alter sterol composition. The diet was supplemented with cholesterol using a modification of the method of Von Elert et al. (Von Elert et al., 2003). Cholesterol was dissolved in ethanol using ultrasonic homogenization (50 W disintegrator with a 3 mm diameter probe at full power for 30 s). After adding the cholesterol to an algal suspension (to yield a supplementation of 0–0.25% of algal dry mass), the suspension was stirred on a rotating platform for 30 m to incorporate the cholesterol.

An initial subsample was taken for size distribution at the start of the experiment. Subsamples were taken after 4 days and 45–90 individuals were digitally photographed for measurement of prosome length. Images were analyzed with ImageJ v. 1.38 (NIH public domain software; http://rsb.info.nih.gov/ij/), using a stage micrometer for calibration, and growth rate calculated as length increment (μm) day^{-1}.

Sample preparation and cholesterol analyses

To expand the scope of the work to include larger numbers of species and sample sizes, whole-animal cholesterol contents were measured. Animals were homogenized, prior to storage in liquid nitrogen (*Acartia hudsonica*, *Calanus finmarchicus* CV) or after freezing (*Acartia tonsa*, *C. finmarchicus* eggs, *Temora longicornis*, *Eurytemora affinis*, *Calanus glacialis*, *Paraeuchaeta norvegica*, *Centropages* spp.), using a 100 µl ground-glass homogenizer with 25 mmol l^{-1} Hepes (pH 7.6) as 2–5% (w/v). Animals were initially pooled to determine wet mass, and then the sample was subdivided to yield homogenates of approximately 2 mg wet mass ml^{-1}. For most species, each subsample consisted of 10–50 individuals. The exceptions were the large *P. norvegica* and *C. glacialis*, for which each sample consisted of two individuals. Levels of cholesterol were determined in triplicate on each subsample using the cholesterol oxidase-based Amplex Red assay (Invitrogen, Carlsbad, CA, USA) with a PerkinElmer LS50B fluorometer equipped with a microplate reader. The addition of cholesterol esterase in the assay allows detection of cholesterol esters as well. However, only free cholesterol (e.g. membrane-associated cholesterol) was detectable using this procedure since cholesterol esters were not at measurable levels. Cholesterol contents were normalized to protein, and protein contents were measured using the Pierce Micro BCA assay (Smith et al., 1985) with bovine serum albumin as standard.

Determination of habitat temperatures

Determining temperature ranges for marine zooplankton can be problematic, as temperatures vary seasonally, geographically and vertically. Individuals may be present well outside their seasonal peak abundances and species may be advected into temperature regimes outside their preferred range. Temperature tolerances determined experimentally can be difficult to compare between studies because of methodological differences. To minimize these limitations, we used two measures to characterize the temperature range of a species, (1) an estimate of maximum habitat temperature the species is likely to encounter in its normal range and (2) experimentally derived temperature tolerance. Temperature tolerance data for *T. longicornis*, *Centropages hamatus* and *C. typicus* are from Halsband-Lenk et al. (Halsband-Lenk et al., 2002), *Calanus glacialis* and *C. finmarchicus* from Hirche (Hirche, 1987) and *E. affinis* from Bradley (Bradley, 1976). Bradley (1976) used a novel, non-destructive bioassay that is not directly comparable to the others, but does support the high temperature tolerance of *E. affinis*. Maximum habitat temperatures were estimated for *Acartia tonsa* and *A. hudsonica* from Sullivan and McManus (Sullivan and McManus, 1986) and for *Temora longicornis* from Halsband-Lenk et al. (Halsband-Lenk et al., 2002). *C. finmarchicus* is often abundant in the 0–50 m depth range in the Gulf of Maine (Clarke and Zinn, 1937) and the maximum habitat temperature for this depth was taken from seasonal data of Gulf of Maine temperatures (Mountain and Jessen, 1987). *Paraeuchaeta norvegica*, a vertically migrating predatory copepod, inhabits the deep water of the Gulf of Maine and its maximum habitat temperature was assumed to be the maximum observed in the 50–100 m depth range. Temperatures at low tide in the estuary where *E. affinis* was collected were measured directly in late July. *Calanus glacialis* is an Arctic shelf copepod (Hirche and Kwasniewski, 1997) and is rare in the Gulf of Maine. It has a more northerly distribution than *C. finmarchicus* (Fleminger and Hulsemann, 1977).

Statistical analyses

A two-way ANOVA and a Bonferroni *post-hoc* test was used to determine the effect of acclimation temperature and species on cholesterol content, as well as effect of acclimation temperature and ontogenic stage (*Calanus* CV copepodites and eggs). Correlation analyses were used to assess the relationship between cholesterol content and habitat temperature (as indicated by maximum habitat temperature or temperature tolerance). $P<0.05$ was considered a statistically significant result. Statistical analyses were performed with Graphpad Prism 5.0 (Graphpad Software, San Diego, CA, USA).

RESULTS

Cholesterol contents and acclimation temperature

Cholesterol contents varied with acclimation temperature in only one out of five species measured (Fig. 1). There was a significant effect of species on cholesterol content (two-way ANOVA, $F=22.4$, $P<0.0001$, d.f.=4; *Calanus* eggs were not used in this analysis) as well as a significant interaction between temperature and species (two-way ANOVA, $F=3.12$, $P=0.0241$, d.f.=4). Only *Calanus* CV copepodites were significantly affected by temperature acclimation, with higher cholesterol content in warm-acclimated animals (Bonferroni *post-hoc* test, $P<0.05$). The other copepod species [*Acartia hudsonica* and *A. tonsa* (Fig. 1B) and *Temora longicornis* and *Eurytemora affinis* (Fig. 1C)] exhibited no change in cholesterol content with acclimation temperature. A separate comparison of *Calanus* eggs and CV copepodites revealed that *Calanus* eggs are significantly enriched in cholesterol compared with copepodites (Fig. 1A; two-way ANOVA, $F=13.7$, $P=0.0012$, d.f.=1), and both eggs and copepodites are enriched in cholesterol at warmer temperatures (two-way ANOVA, $F=30.14$, $P<0.0001$, d.f.=1). There was no interaction between temperature and developmental stage, indicating that both eggs and CVs are affected to the same degree by temperature acclimation (two-way ANOVA, $F=0.0007$, $P=0.980$, d.f.=1).

Dietary cholesterol, temperature and growth

As expected, growth rates were higher in warm-acclimated (25°C) than cold-acclimated (6°C) *Eurytemora affinis*, with maximum growth rates measured at the warm temperature of more than three times that at cold acclimation temperature (Fig. 2). The level of dietary cholesterol affected growth rate most significantly in warm-acclimated animals with maximum growth rates achieved at a higher concentration of cholesterol in the warm-acclimated group than in the cold-acclimated animals. Growth rates at 6°C reached a maximum at <0.05 µg cholesterol l^{-1} whereas <0.2 µg cholesterol l^{-1} is required to realize maximum growth at 25°C.

Cholesterol contents and habitat temperature

A plot of cholesterol contents in copepods *versus* either maximal habitat temperature or temperature tolerance revealed a strong and positive relationship (Fig. 3). Of the species surveyed, copepods that are more commonly found in deeper waters or at higher latitudes (e.g. *Paraeuchaeta norvegica* and *Calanus glacialis*) had the lowest cholesterol levels whereas animals from warmer habitats (e.g. *Acartia tonsa* and *Centropages typicus*) possessed approximately twofold higher amounts of cholesterol. Within genera, the species with the more northerly distributions (*Calanus glacialis*, *Centropages hamatus*, *Acartia hudsonica*) had lower cholesterol levels than congeneric species (*Calanus finmarchicus*, *Centropages typicus*, *Acartia tonsa*) usually found at lower latitudes. Similarly, in the co-familial *Temora lonicornis* and *Eurytemora affinis*, the species with the higher temperature tolerance (*E. affinis*) also contained higher levels of cholesterol. Although no data could be obtained for either maximum habitat temperature or temperature tolerance for *Tortanus discaudatus*, this species had a relatively low cholesterol contents of 4.7 µg cholesterol mg^{-1} protein ($N=6$).

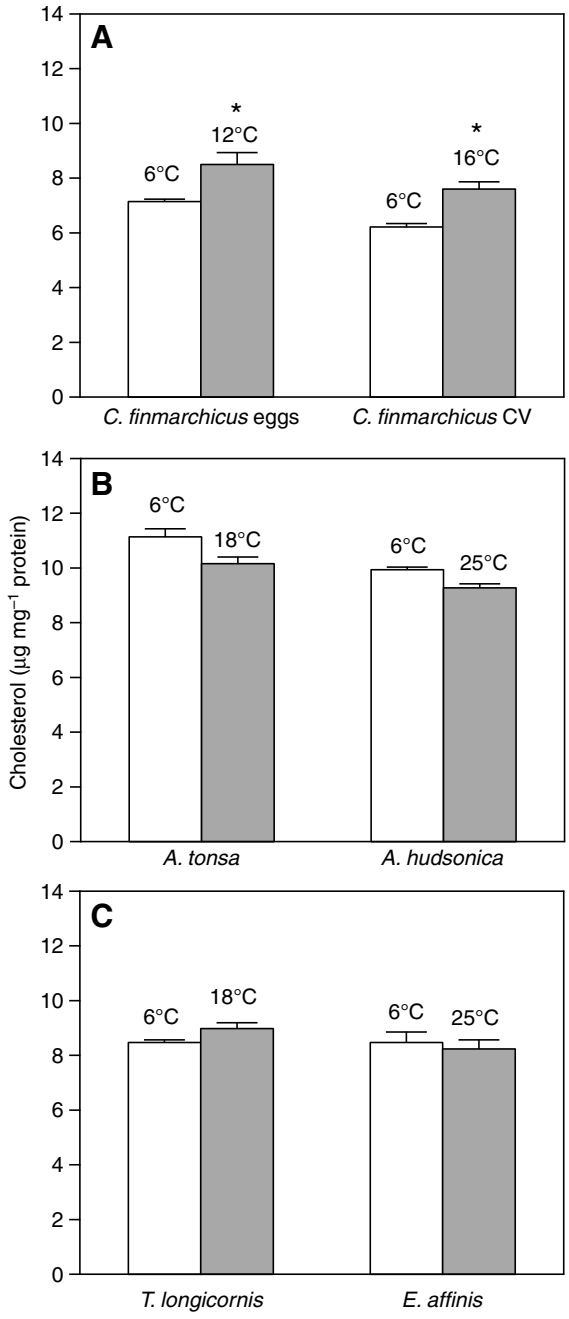

Fig. 1. Effect of acclimation to warm and cold temperatures on cholesterol content of *Calanus finmarchicus* eggs and CV copepodites (A), *Acartia hudsonica* and *Acartia tonsa* (B), *Temora longicornis* and *Eurytemora affinis* (C). Acclimation temperatures are given above columns. Data are means ± s.e.m. *Significant difference at $P<0.05$ (two-way ANOVA). Sample sizes (number of replicate containers per incubator) for each species are: *C. finmarchicus*, $N=6$ (eggs), $N=8$ (copepodites), *A. hudsonica*, $N=2$; *A tonsa*, $N=2$; *T. longicornis*, $N=6$; *E. affinis*, $N=10$. One subsample was taken per replicate container except for *Acartia* spp., for which four subsamples were taken.

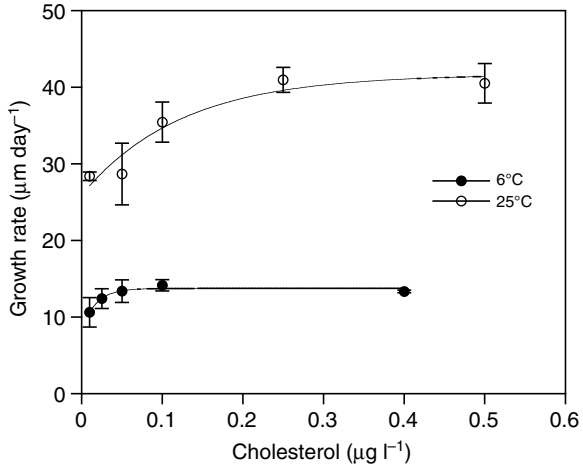

Fig. 2. Growth rates of the copepod *Eurytemora affinis* at 6°C and 25°C at different concentrations of dietary cholesterol. Two replicates were used per treatment, with each replicate calculated from the average length of 45–90 nauplii or copepodites. Data are presented as means ± s.e.m.

DISCUSSION

Cholesterol contents and temperature

The positive correlation between cholesterol levels in copepods and either maximum habitat temperature or temperature tolerance implies that temperature is an important determinant of cholesterol contents in this crustacean group. The relationship between cholesterol content and habitat temperature is strikingly similar for comparisons made both among and within copepod genera (Fig. 3). Cholesterol responses to temperature acclimation, on the other hand, are largely lacking, except for both eggs and copepodites of *Calanus finmarchicus* (Fig. 1). Closer examination of three genera (*Calanus*, *Acartia* and *Centropages*), in which more than a single species was sampled, reveals a pattern corroborating the positive relationship between cholesterol content and habitat temperature. Within a particular genus, the species typically found in colder waters had less cholesterol than congeners common to warmer waters. For example, *Calanus glacialis* is most abundant off the Arctic shelf, whereas *C. finmarchicus* is found throughout the North Atlantic (Conover, 1988). *C. glacialis* contained only 4.8 µg cholesterol mg^{-1} protein whereas *C. finmarchicus* (collected and acclimated at 6°C) has 6.2 µg cholesterol mg^{-1} protein. *Acartia tonsa* is distributed widely from the Gulf of Mexico to the Saint Lawrence River estuary, whereas *A. hudsonica* has the more northerly distribution, extending from Chesapeake Bay to Hudson Bay (Gerber, 2000). Similarly, we observed higher cholesterol contents in *A. tonsa* than in *A. hudsonica*. *Centropages typicus* has a broad temperature tolerance enabling it to extend its distribution from the tropics to the Subarctic (where it overlaps the more northerly *Centropages hamatus*) (Halsband-Lenk et al., 2002). We found the species with the more common occurrence in warmer waters (*C. typicus*) to possess nearly 1.5-times higher levels of cholesterol than its congener (*C. hamatus*). *Tortanus discaudatus*, with its relatively low cholesterol contents (4.7 µg cholesterol mg^{-1} protein), may be somewhat anomalous as this species is found along the entire Atlantic coast and into the Gulf of Mexico. *T. discaudatus*, however, is a late winter/early spring dominant species off the coast of Maine (Gerber, 2000), and its low cholesterol may correspond with its dominance in the colder months.

By contrast, changes in cholesterol content in response to acclimation temperature (a proxy for more short-term changes in temperature) do not present such a strong trend among species of copepods, or in other animals. Our taxa-specific results with temperature acclimations are similar to mixed results from previous studies in fish and crabs (e.g. Crockett and Hazel, 1995; Labbe et

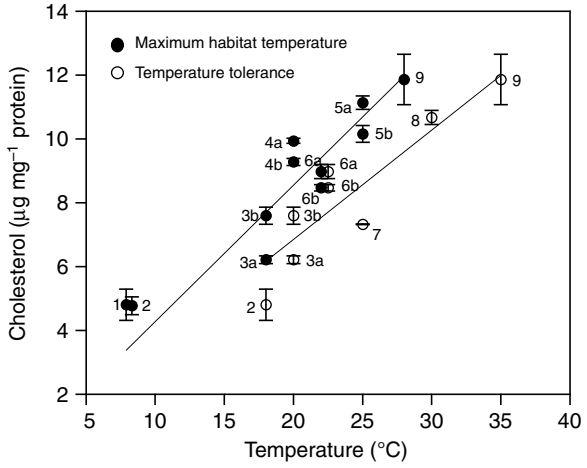

Fig. 3. Relationship between cholesterol content and habitat temperature in copepods. Cholesterol content (µg cholesterol mg^{-1} protein) plotted against both maximum habitat temperatures (filled circles) and temperature tolerance based on LD_{50} (open circles). Species identifications given adjacent to markers: (1) *Euchaeta norvegica*, (2) *Calanus glacialis*, (3a) *Calanus finmarchicus* cold acclimated, (3b) *C. finmarchicus* warm acclimated, (4a) *Acartia hudsonica* cold acclimated, (4b) *A. hudsonica* warm acclimated, (5a) *Acartia tonsa* cold acclimated, (5b) *A. tonsa* warm acclimated, (6a) *Temora longicornis* cold acclimated, (6b) *T. longicornis* warm acclimated, (7) *Centropages hamatus*, (8) *Centropages typicus*, (9) *Eurytemora affinis*. To conform to the conditions experienced by the other copepods, *E. affinis* was acclimated to seawater (32 p.p.t.) for 7 days. Data are presented as mean ± s.e.m. Sample sizes are N=10 for *E. norvegica*, *C. typicus* and *E. affinis*; N=8 for *C. finmarchicus*, N=6 for *C. glacialis* and *T. longicornis*, and N=2 for *A. hudsonica*, *A. tonsa*, and *C. hamatus*. Correlation analysis of temperature tolerance, r^2=0.82, significance level P<0.001, maximum habitat temperature r^2=0.84, significance level P<0.001.

al., 1995; Robertson and Hazel, 1995; Cuculescu et al., 1995). Acclimatory responses in cholesterol contents are more likely to depend on the species in question (this study), particular tissues (e.g. Robertson and Hazel, 1995; Labbe et al., 1995; Cuculescu et al., 1995), the specific membrane being analyzed (Crockett and Hazel, 1995), and even the lipid microdomain within the membrane (Zehmer and Hazel, 2003). When taken together, these studies indicate that changes in body temperature over timescales of days and/or weeks may, but also may not, result in adjustments of cholesterol levels. In the instance when cholesterol content did increase with warm acclimation (*Calanus finmarchicus*), the response was consistent with the role of cholesterol in modulating membrane fluidity.

It is possible that insufficient time was allowed for acclimation of membrane cholesterol content to occur in *Acartia* spp., *Temora* and *Eurytemora*, which were acclimated for 7 days. However, Bradley (Bradley, 1978) determined that full temperature tolerance in *Eurytemora affinis* occurred in 3–4 days, and 7 days represents a significant portion of the development time of these animals (approximately 15–25 days) (Mauchline, 1998). Since *Calanus finmarchicus* demonstrates an acclimation response and has a development time two to three times longer than the other species used in this study, we believe it is unlikely that longer acclimation times would yield different results.

Not only is the association between cholesterol content and habitat temperature more well-defined than that of acclimation temperature, the absolute differences in cholesterol contents are also much greater for copepods adapted to various thermal regimes than differences when copepods are acclimated to cold and warm temperatures. For the species sampled, the highest and lowest maximum habitat temperatures (or temperature tolerances) differ by a factor of at least two, and cholesterol contents follow a similar trajectory – species living at somewhat warmer temperatures (e.g. *Acartia tonsa*, *Centropages typicus*) have twice the cholesterol content of those species commonly found in cold-water habitats (e.g. *Euchaeta norvegica* and *Calanus glacialis*). These very substantive differences in cholesterol contents stand in sharp contrast to those we observed with the temperature acclimations. Although the temperatures used in laboratory acclimations have a range of 2.5- to 4-fold (i.e. at least as great as the range of habitat temperatures for the species sampled), either there is no significant effect on cholesterol content (*Acartia* spp., *Temora* and *Eurytemora*), or only a relatively modest (10–20%) change occurs (*Calanus finmarchicus*).

Cholesterol content appears to be subject to habitat temperature in another crustacean group, and in animals more generally. In the warm-tolerant cladoceran, *Daphnia magna* Straus, cholesterol content is significantly higher (<18 µg cholesterol mg^{-1} protein; R.P.H. and E.L.C., unpublished) than in any of the copepods measured in the present study. By contrast with *D. magna*, the cold-temperate cladoceran *Pleopis polyphemoides* Leuckart has cholesterol contents that are only about half that of *D. magna* (8.5 µg cholesterol mg^{-1} protein; R.P.H. and E.L.C., unpublished). The suggestion that body temperature underlies, at least in part, the cholesterol content of animals has been made previously (Robertson and Hazel, 1997). Using data compiled from several studies, including those of two ectotherms (trout, tortoise), a bird and several mammals, the authors suggest the higher levels of cholesterol in membranes from endotherms (compared with those levels found in ectotherms) counter the fluidizing effects of warm body temperatures. Although in the present work whole-animal cholesterol contents were measured, it is probable that the cholesterol we have quantified largely reflects membrane cholesterol since in an earlier study (Crockett and Hassett, 2005) similar trends were obtained for crude homogenates and biological membranes. Taken together, the data from closely related crustaceans (current study) with those reported from vertebrates, provide compelling evidence for cholesterol's stabilizing role at different body temperatures.

Elevated cholesterol levels in copepods may enable these animals to extend their range into warmer habitats since animals with the greatest temperature tolerances possess the highest cholesterol contents. Cholesterol, however, may also protect ectothermic animals against cold shock injury, since modulating the cholesterol content by dietary means confers additional protection in *Drosophila* (Shreve et al., 2007). Cholesterol could also play a part in temperature tolerances of different populations within a species. Although each copepod species used in the current study is likely to represent a single population, population-level differences in temperature tolerances of copepods have been documented [*Acartia tonsa* (González, 1974); *Centropages typicus* (Halsband-Lenk et al., 2002); as well as the cladoceran *Daphnia* (MacIsaac et al., 1985)]. Although we do not have data on cholesterol content of copepod populations in waters warmer than the Gulf of Maine, given the trends we observe among species, we might expect population-level differences in cholesterol content as well. It is also worth noting that egg development rates of *Calanus finmarchicus* differ between samples taken from a Norwegian fjord during two different years yet raised at the same low temperature (Pederson and Tande, 1992). The authors attribute the differences to markedly different temperatures during the overwintering period between the two years (1–1.5°C in 1980 *vs* 4–5°C in 1989), and speculate that the

overwintering temperature experienced by *C. finmarchicus* could affect the temperature tolerance of the offspring. Our own data indicate that the cholesterol contents of both CV copepodites (the overwintering stage) and eggs of *C. finmarchicus* change during temperature acclimation, providing at least a partial physiological basis for these observations.

Cholesterol feeding, growth and temperature

Cholesterol supplementation enhances growth of *Eurytemora affinis* at either cold (6°C) or warm (25°C) temperature with a higher incipient limiting concentration at the warmer temperature. Although temperature effects were not determined, a similar enhancement of growth rates was also observed in the cladoceran *Daphnia galeata* when the cyanobacterial diet was supplemented with cholesterol (Von Elert et al., 2003). Why are growth rates limited at a higher cholesterol concentration in warm-acclimated *Eurytemora*? Since cholesterol contents of *E. affinis* acclimated to 6° and 25°C are comparable (Fig. 1C), the differences in the growth curves are not due to different body cholesterol content. It is quite possible that at 6°C *Eurytemora* is temperature limited under these food conditions, and only when cholesterol content is extremely low does sterol limitation supercede temperature limitation. Thus an increase in temperature from 6°C to 25°C at other cholesterol concentrations would shift the animals from temperature-limited to sterol-limited growth. Changes in the incipient limiting concentration may also be affected by assimilation and/or processing of cholesterol. Higher growth rates at warm temperatures necessitate increased demand for cholesterol along with other elemental nutrients (e.g. C, N, P). Limiting concentrations of cholesterol relative to these other nutrients should not change, unless these different dietary components are assimilated or processed differently. If cholesterol is assimilated less efficiently than other potentially limiting nutrients, the incipient limiting concentration may increase with ingestion rate, since an excess of the more efficiently assimilated nutrients will tend to accumulate.

Although the literature is limited, there is evidence that phytosterols are assimilated less efficiently than other components of a copepod's diet. Phytosterol assimilation in *Calanus helgolandicus* ranges from negligible up to nearly 60% depending upon the phytosterol in question (Harvey et al., 1987). By contrast, fatty acids are assimilated at over 90% efficiency. In the cladoceran *Daphnia galeata* growth rates on diets supplemented with preferred phytosterols (e.g. stigmasterol, sitosterol, ergosterol) are comparable to those supplemented with cholesterol, whereas supplementation with other sterols (dihydrocholesterol, lanosterol) yield poor growth rates (Martin-Creuzburg and Von Elert, 2004).

The effect of environmental conditions (i.e. food concentration and quality, temperature and salinity) on assimilation of sterols in copepods is not well understood. Processing of dietary phytosterols will involve both assimilation and metabolic de-alkylation, both of which can vary (Knauer et al., 1999; Harvey et al., 1987; Teshima, 1971). Cholesterol and phytosterols are taken up directly by midgut cells in insects (Canovoso et al., 2001; Jouni et al., 2002) and de-alkylation of phytosterols then takes place within the midgut cells, rather than in the gut lumen. Sterol assimilation in *Calanus helgolandicus* is influenced by food concentration, with higher assimilation efficiencies at low food concentrations (Harvey et al., 1987), a pattern also observed by Landry et al. (Landry et al., 1984) for carbon and nitrogen assimilation efficiency in *C. pacificus*. Differences in assimilation efficiency may be ascribed to differences in residence time in the gut or in rates of uptake into gut cells. Gut residence times of copepods generally are inversely related to food concentration (Dagg and Walser, 1987; Besiktepe and Dam, 2002; Tirelli and Mayzaud, 2005) although some studies have not found such a relationship (e.g. Ellis and Small, 1989). Longer gut residence times may allow more efficient assimilation of phytosterols observed in *C. helgolandicus* at low food concentrations (Harvey et al., 1987), particularly if phytosterols are more difficult to assimilate. Gut residence times also are inversely related to temperature (Dam and Peterson, 1988), and similarly may lead to less efficient assimilation of cholesterol at high temperatures due to rapid gut transit, consistent with results observed with *Eurytemora affinis* (Fig. 2). One might then expect sterol limitation to be more pronounced when some combination of low algal sterol content, warm temperatures, and high algal concentrations occur.

Conclusions

The positive relationship in copepods between cholesterol content and habitat temperature points to a significant role for temperature in setting cholesterol levels in animals more generally than has been previously recognized. Our results, combined with comparisons made for ectothermic and endothermic vertebrates (Robertson and Hazel, 1997), are strong evidence for cholesterol stabilizing membranes over a wide range of body temperatures. For copepods, and many invertebrates that must acquire sterol exogenously, an animal's demand for dietary sterol is likely to vary with temperature. Given the taxa-specific responses to temperature acclimation, however, it is not possible to generalize about how temperature changes over the short-term affect cholesterol content, and hence cholesterol demand, in copepods. Although elevated temperature increases the proportion of dietary cholesterol needed to maximize growth rates in *Eurytemora affinis*, the cholesterol content of *Eurytemora* is not altered, indicating that either growth rates at low temperature are temperature limited except at very low cholesterol concentrations, or cholesterol assimilation and/or turnover is(are) temperature-dependent process(es).

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