

# Effect of Temperature on the Fatty Acid Composition and Temporal Trajectories of Fatty Acids in Fasting *Daphnia pulex* (Crustacea, Cladocera)

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**ABSTRACT:** Poikilothermic organisms accumulate highly unsaturated FA (HUFA) in their lipids at reduced temperatures to maintain cell membrane fluidity. In this study we investigated the effect of temperature on temporal trajectories of FA of fasting *Daphnia pulex* cultured on a HUFA-free diet. *Daphnia pulex* populations were maintained for 1 mon at 22 and 11°C and were fed the chlorophyte *Ankistrodesmus falcatus*. We observed conversion of C<sub>18</sub> FA precursors to EPA (20:5n3) and arachidonic acid (ARA; 20:4n6) in *D. pulex*. We showed that long-term exposure to cold temperature causes a significant increase in EPA. HUFA such as ARA and EPA are highly conserved during starvation. Therefore, *D. pulex* has the biosynthetic capacity to adjust and to maintain the content of HUFA required to survive at low temperatures.

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Species in the genus *Daphnia* are eurythermal organisms with a wide geographical distribution (1) that exposes them to dramatic changes in temperature. *Daphnia pulex*, for example, can be cultivated at constant temperatures ranging between 2°C and 30°C (2). Many poikilothermic animals adapt to changing environmental temperatures by modifying the degree of unsaturation of their lipids (3,4). Highly unsaturated FA (HUFA) are defined as PUFA that have more than three double bonds and carbon chain lengths of C<sub>20</sub> or longer (5). The high degree of unsaturation of HUFA imparts to them the property of low m.p. The accumulation of HUFA in the lipids of poikilothermic organisms at reduced temperatures can therefore be explained as an adaptation to maintain cell membrane fluidity and thus membrane function.

When *Daphnia* feed on diets containing HUFA, for example, EPA (20:5n3) or arachidonic acid (ARA; 20:4n6), their lipids become enriched with these FA (6,7). However, *Daphnia* appear unable to accumulate large amounts of docosahexaenoic acid (DHA; 22:6n3), which they readily convert to EPA (6,8). *Daphnia* can use  $\alpha$ -linolenic acid (ALA; 18:3n3) and linoleic acid (LIN; 18:2n6) as precursors for the synthe-

sis of EPA and ARA, respectively (6,9,10). However, *de novo* rates of FA synthesis in *Daphnia* have been shown to be less than 2% (11).

Short-term (48 h) exposure to cold temperatures (4–5°C) did not cause appreciable changes in HUFA levels of *D. pulex* (8) or *D. magna* (9). These observations indicate either that *Daphnia* exposed to cold temperatures must be highly dependent on dietary HUFA, or that enzymes involved in the HUFA synthesis need longer adaptation periods to reach full activity. We hypothesize that *D. pulex* has the biosynthetic capacity to adjust and maintain the HUFA content necessary to survive at low temperatures. Bychek *et al.* (12) recently showed that cultures of *D. magna* are able to tolerate brief (24 h) spells of fasting with very little change in lipid metabolism. Here we investigate the effect of temperature on temporal trajectories of FA of fasting *D. pulex* (hereafter referred to as *Daphnia*) grown exclusively on a HUFA-free diet.

## MATERIAL AND METHODS

**Culture conditions.** The chlorophyte *Ankistrodesmus falcatus* (AF; strain UTCC63) was cultured in modified CHU-10 medium (13). Algae were cultured in semicontinuous chemostats at 22°C under both a General Electric Cool-White® 40-watt and a Phillips F40T12 40-watt Plant-and-Aquarium® fluorescent lamp with a combined emission of 220  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation. Specimens of *Daphnia pulex* (Carolina Biological Supply Company, Burlington, NC) were cultured in glass aquaria at 22°C (DA22) or 11°C (DA11) under a 12-h light–12-h dark cycle, and the *D. pulex* were fed *ad libitum* with AF that were concentrated by centrifugation before feeding. *Daphnia* were maintained under these constant conditions for 1 mon prior to their use in starvation experiments.

**Starvation experiments.** Tap water was preconditioned to remove chlorine and ammonia and was filtered through a 0.2- $\mu\text{m}$  Sartorius membrane filter to remove most bacteria. *Daphnia* were separated from algae by pouring them onto a 350- $\mu\text{m}$  mesh nylon sieve and by repeatedly rinsing them with filtered water to finish the cleaning process. Clean *Daphnia* were kept in filtered water for 24 h to provide a standard time for hind-gut clearance. *Daphnia* were counted and distributed equally among 500-mL flasks filled with 400 mL of filtered

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Abbreviations: AF, *Ankistrodesmus falcatus*; ALA,  $\alpha$ -linolenic acid; ARA, arachidonic acid; DA11, *Daphnia pulex* cultured at 11°C; DA22, *Daphnia pulex* cultured at 22°C; HUFA, highly unsaturated FA; LIN, linoleic acid; MUFA, monounsaturated FA; TFA, total FA content.

water to obtain homogenous groups. Nongravid mid-sized animals (subadults) were selected to avoid the release of neonates during the experiment (14). *Daphnia* that were pre-adapted to 11 and 22°C for 1 mon had a similar average dry weight on Day 0 of the starvation experiment (17.7 and 18.1 µg/animal, respectively). Twenty-one flasks were stocked with *Daphnia* that were pre-adapted to 11°C (75 animals/flask). Twenty-four flasks were stocked with animals that were pre-adapted to 22°C (100 animals/flask). *Daphnia* were starved at original culture temperatures under constant dim fluorescent lighting conditions. Each day, three flasks were chosen randomly, and live *Daphnia* were collected from them. All animals were counted to determine the survival rate. When the survival rate fell below 50%, the experiments were terminated to guarantee a sufficient tissue mass for FAME analysis. DA22 could be collected only from 12 flasks because the survival rate decreased abruptly to 16.4% on Day 4. The average survival rate for DA11 was 85.8% on Day 6. *Daphnia* were frozen (at -85°C) after removing as much water as possible so as to minimize bacterial contamination, then they were freeze-dried and weighed prior to FA analysis.

**FAME analyses.** FAME of algae and *Daphnia* were extracted by grinding freeze-dried tissues in 2:1 (vol/vol) chloroform/methanol (15). FA were transmethylated with boron trifluoride in methanol (14% w/w). FAME concentrations were quantified on a Hewlett-Packard 6890 gas chromatograph with the following configuration: splitless injection; column = Supelco SP-2560 100 m × 0.25 mm i.d. × 0.20 µm thick film; oven = 140°C (hold for 5 min), then to 240°C at 4°C min<sup>-1</sup>, hold for 12 min; carrier gas = helium, 1.2 mL/min; detector = FID at 260°C; injector = 260°C; run time = 42 min/sample.

**Statistical analysis.** All results are presented as mean ± SE. Independent samples collected on Day0/1, Day2/3, and Day4/5 were grouped prior to one-way ANOVA. ANOVA was followed by a Tukey test when a significant ( $P < 0.05$ ) difference was found. Percentage data were arcsine transformed (16).

## RESULTS AND DISCUSSION

**Effects of growth temperature on the FA composition of *Daphnia* that were fed *Ankistrodesmus falcatus*.** The FA compositions of AF, DA11, and DA22 are presented in Table 1. The lipid content of AF was 26% of its dry weight. HUFA as ARA, EPA, or DHA could not be detected in lipids extracted from AF. However, with 44.3% ALA and 15.9% LIN, total FA content (TFA) of AF contained two important precursors required for the synthesis of HUFA. AF was therefore a suitable diet to investigate the biosynthetic capacity for HUFA in *Daphnia*. In contrast to their diet, *Daphnia* grown at 11 and 22°C contained HUFA (ARA and EPA). The content of EPA was clearly influenced by temperature, because a significantly higher level of EPA was found in DA11 (12.7%) compared with DA22 (3.1%). *Daphnia* are clearly able to increase the proportion of EPA in their lipids to maintain cell membrane fluidity at low temperatures. However,

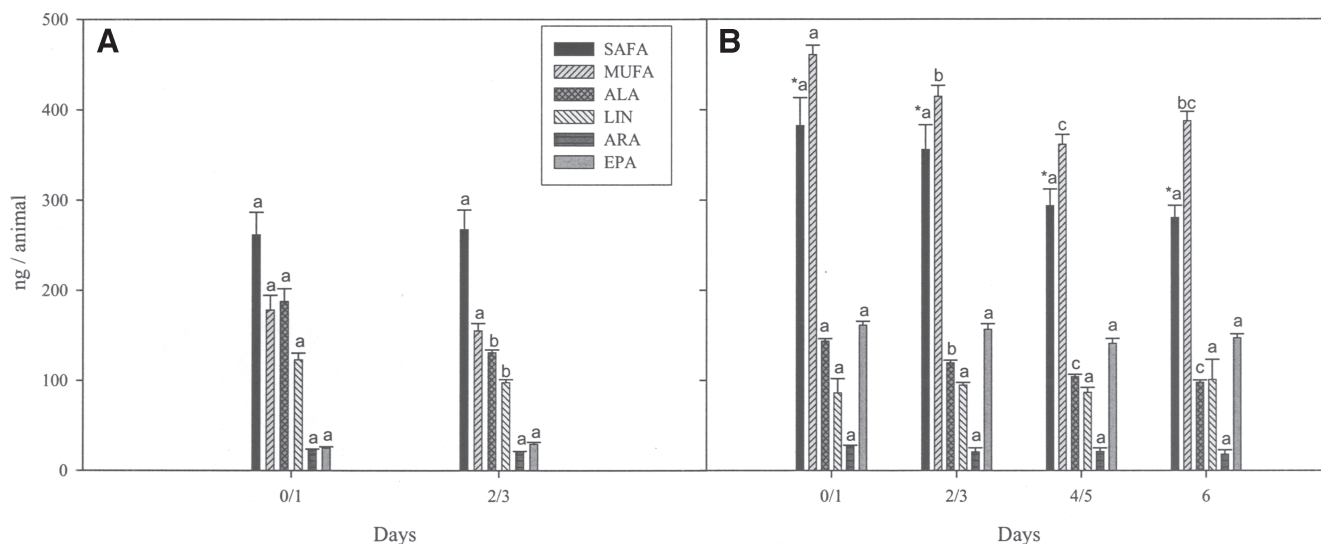
**TABLE 1**  
Effects of Growth Temperature on FA Composition (% of total identified FAME) of *Daphnia pulex* Fed *Ankistrodesmus falcatus*

	<i>A. falcatus</i>	DA22		DA11	
		Mean	SE	Mean	SE
11:0	nd	nd		0.3	0.2
12:0	0.6	0.2	0.0 <sup>a</sup>	1.0	0.2 <sup>a</sup>
14:0	0.3	1.4	0.1 <sup>a</sup>	1.7	0.1 <sup>a</sup>
15:0	0.1	0.8	0.0 <sup>a</sup>	0.5	0.0 <sup>b</sup>
16:0	20.4	18.6	0.5 <sup>a</sup>	19.1	0.6 <sup>a</sup>
16:1n-7	1.0	3.3	0.1 <sup>b</sup>	13.0	0.4 <sup>a</sup>
17:0	0.1	0.9	0.0 <sup>a</sup>	1.1	0.0 <sup>a</sup>
18:0	0.5	8.1	0.5 <sup>a</sup>	6.2	1.7 <sup>a</sup>
18:1n-9t	nd	0.1	0.0 <sup>a</sup>	0.1	0.0 <sup>a</sup>
18:1n-9c	11.6	19.2	0.5 <sup>b</sup>	23.2	0.5 <sup>a</sup>
18:2n-6c	15.9	14.3	0.3 <sup>a</sup>	6.8	1.2 <sup>b</sup>
20:0	nd	0.3	0.1	nd	
18:3n-6	1.4	1.2	0.0 <sup>a</sup>	0.7	0.0 <sup>b</sup>
20:1n-9	nd	0.1	0.0	nd	
18:3n-3	44.3	22.4	0.3 <sup>a</sup>	11.3	0.2 <sup>b</sup>
21:0	nd	0.1	0.0	nd	
20:2	nd	1.8	0.2	nd	
22:0	2.4	0.5	0.1	nd	
20:3n-6	nd	0.1	0.0 <sup>a</sup>	0.1	0.0 <sup>a</sup>
22:1n-9	nd	0.3	0.0	nd	
20:3n-3	nd	0.2	0.0	nd	
20:4n-6	nd	2.7	0.3 <sup>a</sup>	2.1	0.1 <sup>a</sup>
24:0	0.9	0.2	0.0	nd	
20:5n-3	nd	3.1	0.2 <sup>a</sup>	12.7	0.4 <sup>b</sup>
24:1n-9	0.2	0.3	0.3	nd	
Σn-3	44.3	25.6	0.3 <sup>a</sup>	24.0	0.6 <sup>a</sup>
Σn-6	17.4	18.2	0.5 <sup>a</sup>	9.7	1.3 <sup>b</sup>
ΣSAFA	25.4	31.0	1.0 <sup>a</sup>	30.0	2.2 <sup>a</sup>
ΣMUFA	12.9	23.3	0.4 <sup>b</sup>	36.3	0.4 <sup>a</sup>
ΣPUFA	61.7	45.7	0.5 <sup>a</sup>	33.7	1.8 <sup>b</sup>

<sup>a</sup>Results are means ± SE. Values within a row with a different superscripted letter are significantly different ( $P < 0.05$ ). nd = not detected. DA22, *Daphnia pulex* cultured at 22°C; DA11, *D. pulex* cultured at 11°C; SAFA, saturated FA; MUFA, monounsaturated FA.

for optimal growth and reproduction, additional dietary EPA might be required (17,18).

ARA is an important precursor for signaling molecules including prostaglandins, prostacyclins, and thromboxanes (5). In contrast to EPA, DA11 and DA22 contained similar levels of ARA (2–3%). Therefore, the biosynthesis of ARA is unaffected by the difference in temperature within this range. We suggest that EPA and ARA were produced by conversion of dietary LIN and ALA precursors, especially at the lower temperature. In nature, *Daphnia* might therefore play an important role in trophic upgrading (19) of FA, thereby improving the nutritional quality of algae for subsequent use by higher trophic levels (e.g., invertebrate predators and fish). We cannot exclude the possibility that ultratrace amounts of dietary HUFA or HUFA of bacterial origin (gut bacteria) were accumulated by *Daphnia*. However, it is unlikely that EPA, at levels greater than 10% of TFA, would be derived from bacteria, particularly as care was taken to remove bacteria from the water and to clear the gut of the *Daphnia*. DHA was not detected in these *D. pulex*, confirming the results of Bychek *et al.* (12) and Farkas *et al.* (9) on *D. magna*.



**FIG. 1.** Temporal trajectories of FA in fasting *Daphnia pulex* kept at (A) 22 and (B) 11°C. Results are means  $\pm$  SE. Significant differences between means were determined by one-way ANOVA followed by Tukey's multiple comparison test. Values of the same FA with a different superscripted letter are significantly different ( $P < 0.05$ ). An asterisk indicates that there was a statistically significant difference ( $P = 0.031$ ), but the power of the performed test (0.56) was below the desired power of 0.80. ALA,  $\alpha$ -linolenic acid; ARA, arachidonic acid; LIN, linoleic acid; MUFA, monounsaturated FA; SAFA, saturated FA.

DA11 had a significantly higher content of monounsaturated FA (MUFA), 36.3%, compared with DA22, which had 23.3%. The higher level of MUFA in DA11 might be explained by cold-induced expression of  $\Delta 9$ -desaturase as observed in carp (20), and can be described as a further adaptation to maintain cell membrane fluidity because MUFA have a significantly lower m.p. compared with their saturated analogs (21).

*Effect of temperature on temporal trajectories of FA in fasting Daphnia.* DA11 had a higher TFA on Day 0/1 (1272 ng/animal) compared with DA22 (821 ng/animal). The lower TFA in DA22 might be explained by the higher metabolic rate compared with DA11 ( $Q_{10} = 2$ ; ref. 22). The term  $Q_{10}$  is defined as the increase in the rate of metabolic activity caused by a 10°C increase in temperature (23). If the rate doubles,  $Q_{10}$  is 2. TFA values decreased during starvation to 1061 and 779 ng/animal in DA11 and DA22, respectively. The insignificant decrease of TFA in DA22 during starvation suggests that only minor lipid reserves were available, which is confirmed by the high mortality of these animals within the first 3 d. ARA and EPA are important structural components of membrane glycerolipids, and were highly conserved during starvation in both DA11 and DA22 (Fig. 1). This agrees with Kainz *et al.* (24), who noted that EPA and ARA are highly retained in zooplankton. ALA decreased significantly in both treatments after being metabolized in order to resist starvation or after conversion to EPA. A significant decrease was also observed for LIN in DA22; however, no changes were observed in DA11.

In nature, periods of food shortage occur that can, temporarily, result in starvation conditions for zooplankton (25–27). Several effects of starvation on the biochemical composition of *Daphnia* have been described (14,28). However, we show, for the first time, trajectories of FA concentra-

tions in starved animals under different temperature regimes. After further laboratory calibration, characteristic levels of selected FA in fasting *Daphnia* might be better defined, providing more quantitative assessments about the condition of field-derived *Daphnia* specimens.

FA can be used as trophic markers of dietary composition (29). However, our data indicate that such studies require detailed information about the effect of temperature on the extent of dietary FA conversion in different organisms.

*Conclusions.* We showed that long-term exposure of *D. pulex* to cold temperature causes a significant increase in EPA. HUFA, such as ARA and EPA, are highly conserved during starvation. Therefore, *D. pulex* has the biosynthetic capacity to adjust and to maintain the content of HUFA required to survive at low temperatures.

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