

Differences in heat tolerance within a *Daphnia magna* population: the significance of body PUFA content

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Abstract In this study, we use the model organism *Daphnia magna* to determine variation in heat tolerance within a natural population and relate this to variation in body fatty acid content and composition. As a proxy for heat tolerance we measured time to immobilization (T_{imm}) for 25 clonal lineages isolated from a single lake. The clonal lineages were grown on *Chlamydomonas reinhardtii* at 15°C. Upon deposition of the first clutch, T_{imm} and body fatty acids were determined. We report significant differences of T_{imm} and of the content of PUFAs and of eicosapentaenoic acid (EPA) among the *D. magna* clonal lineages. T_{imm} was negatively related to total PUFA-content, indicating that increased body PUFA-content may be detrimental for membrane properties at high temperatures. Juvenile somatic growth varied significantly among clonal lineages, and was not related to total body PUFA-content, hence body PUFA-content was no proxy for PUFA-limitation. No correlative evidence for an impact of body EPA-content on T_{imm} or juvenile growth was obtained. We hypothesize that the observed intra-population variability of body PUFA-

content might be related to potentially different amplitudes in diel vertical migration in response to fish. These different amplitudes would mean differences in the extent of diurnal temperature fluctuations among the clonal lineages.

Keywords Temperature · *Daphnia* · Heat tolerance · EPA · Polyunsaturated fatty acid

Introduction

During the past 50 years mean global temperatures have increased substantially, which has affected species ranges and the response to seasonal events, as is reflected in timing of migration or flowering (Parmesan & Yohe, 2003; Bradshaw & Holzapfel, 2006). Although aquatic populations may in principle adapt to increased warming (e.g., Schluter et al., 2014; Geerts et al., 2015), such ability to evolve requires a standing stock of variation in the respective populations with regard to temperature tolerance.

Temperature tolerance in poikilotherms usually is assessed by reaction norms of the growth rate as a function of temperature, which provide information about optimal physiological temperatures. In addition to thermal performance curves also measures of heat tolerance have been used to predict organisms' responses to climate change (Helmuth et al., 2005;

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Sinclair et al., 2016). A widely used index of heat tolerance is the critical thermal maximum (CT_{Max}), which is the upper temperature at which animals lose motor function (Huey et al., 2012). Hence, CT_{Max} determines the short-term ability to tolerate extreme temperatures. CT_{Max} has proven helpful for predicting responses to global warming in several species (Huey et al., 2012), and in *Drosophila* CT_{Max} has been shown to partly reflect field fitness (Kristensen et al., 2007).

Daphnia are major consumers of phytoplankton, and several correlative studies have shown that the dietary availability of the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA, C20:5n-3) in natural phytoplankton is a very strong predictor of *Daphnia* growth (Müller-Navarra, 1995; Müller-Navarra et al., 2000; Wacker & von Elert, 2001). However, Müller-Navarra (1995) and Wacker & von Elert (2001) reported similarly high predictive power for several other n-3 and n-6 PUFAs, which was due to strong inter-correlations among different PUFAs in natural phytoplankton, so that an identification of a single limiting PUFA based on correlations seemed not convincing. These evidences and the finding that the content of EPA and of other n-3 PUFAs in phytoplankton decreases with nutrient loading of lakes and ponds (Müller-Navarra et al., 2004; Persson et al., 2007) suggest that PUFA-limitation of *Daphnia* may be widespread. For EPA, supplementation experiments have confirmed that growth and reproduction of *Daphnia* may be limited by a low EPA-content of its diet (e.g., von Elert, 2002; Becker & Boersma, 2005; Martin-Creuzburg et al., 2010, 2012; Windisch & Fink, 2018). This limitation by EPA and other PUFAs is well explained by the fact that crustaceans, like all arthropods, are incapable of synthesizing long-chain PUFAs de novo and therefore require a dietary source of these lipids to satisfy their physiological demands (Harrison, 1990). Long-chain PUFAs may be synthesized by elongation and desaturation of PUFAs with shorter carbon chain length; although in several cases the rates have been shown to be too low to meet demands (Weers et al., 1997; von Elert, 2002; Taipale et al., 2011). PUFAs are important constituents of cell membranes and may furthermore serve as precursors for eicosanoid biosynthesis and immune functions in *Daphnia* (Schlotz et al., 2012, 2016).

In a seminal paper, using a PUFA-free diet, Martin-Creuzburg et al. (2012) have demonstrated that population growth rates of *Daphnia magna* STRAUS

are limited by a low availability of PUFAs at low (10°C) but not at higher temperatures and that the supplementation with several single PUFAs shifted the temperature optimum for offspring production to colder temperatures. In line with these temperature effects on reaction norms, supplementation with EPA has been shown to increase juvenile growth at 15°C but not at 20°C when *D. magna* was fed the green algae *Acutodesmus obliquus* (Sperfeld & Wacker, 2012) or *Chlamydomonas klinobasis* (von Elert & Fink, 2018). Although many *Daphnia* species escape winter temperatures by forming resting eggs (Sommer et al., 1986), they still have to cope with distinct temperature fluctuations, e.g., during diel vertical migration (Stich & Lampert, 1981).

Both, reaction norms and heat tolerance (CT_{Max}), have been applied to study temperature tolerance of *Daphnia* in ex-ephippia clones (Mitchell & Lampert, 2000; Geerts et al., 2015), and heat tolerance in *Daphnia* has been estimated as time to immobilization (T_{imm}) at a temperature deadly in the longer run (37°C, Williams et al., 2012; Yampolsky et al., 2014; Coggins et al., 2017). Although temperature tolerance in *Daphnia*, determined as reaction norms, has been shown to vary among ex-ephippia clones from the same location (Mitchell & Lampert, 2000), intra-population variation in coexisting *Daphnia* genotypes has neither been determined with respect to temperature tolerance nor to heat tolerance.

Based on the earlier findings of Martin-Creuzburg et al. (2012) and von Elert & Fink (2018), we here have grown *D. magna* under putatively PUFA-limiting conditions, i.e., at 15°C on *C. klinobasis*. We hypothesized that heat tolerance varies within a natural *Daphnia magna* population and that heat tolerance is related to variation in the PUFA-content among clonal lineages. We therefore assessed heat tolerance as knock-out time (time until immobilization, T_{imm}) and investigated its correlation with the PUFA-content of the different *D. magna* clonal lineages.

Methods

Test species and cultures

From May to July 2010, monthly samples of living *Daphnia magna* STRAUS were collected from Lake Bysjön (situated in Southern Sweden: N 55.675399 E

13.545805) from a depth of 1–2 m with a plankton net (200 μm). The animals were cultivated in clonal lineages (Schwarzenberger et al., 2013), of which 25 were available when these experiments were performed. Animals were kept for several generations at 20°C in aged, membrane-filtered (pore size: 0.45 μm) tap water under dim light.

Under standard conditions, no more than 15 animals per liter were kept under non-limiting food concentrations of 2 mg $C_{\text{part}} l^{-1}$ of the green alga *Chlamydomonas klinobasis*, strain 56, culture collection of the Limnological Institute at the University of Konstanz. *C. klinobasis* was grown in 5 l semi-continuous batch cultures (20°C; light intensity 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$) by replacing 20% of the culture with fresh, sterile Cyano medium (von Elert & Jüttner, 1997) with vitamins (thiamine hydrochloride 300 nM, biotin 2 nM, and cyanocobalamine–vitamin B12 0.4 nM) every other day. The test animals originated from mothers that had been raised under control conditions (saturating concentrations of *C. klinobasis*) for at least five generations.

Growth experiments

When *D. magna*, maintained under standard conditions at 20°C, produced their first clutch of eggs, these animals were transferred to 15°C with identical feeding conditions. Growth experiments were initiated with newbornes (< 16 h old) from the third clutch of these clonal mothers maintained at 15°C. The experiments were carried out in glass beakers filled with 250 ml aged and filtered (0.45 μm filter) tap water and containing nine to twelve individuals of *D. magna*. Experiments with each clonal lineage were carried out in triplicate. The animals were transferred into fresh food suspensions every other days until they produced their first clutch of eggs. Then two individuals per replicate were removed for dry mass determination. Further three or four individuals per replicate were removed for fatty acid analysis, and the remaining individuals were used for the analysis of T_{imm} (see below). The somatic growth rate (g) was calculated according to the formula: $g = (\ln W_t - \ln W_0) \times t^{-1}$, where W_0 is the initial dry mass of neonates, W_t is the mass of the individual at the end of experiment, and t is the duration of the experiment (Wacker & von Elert, 2001). For the determination of the initial dry mass, subsamples of ten individuals were used.

Time until immobilization (T_{imm})

Time until immobilization (T_{imm}) was determined as an estimate of temperature tolerance at 37°C for each *D. magna* clonal lineage (Williams et al., 2012; Yampolsky et al., 2014). Newbornes of the third clutch of each *D. magna* clonal lineage obtained at 15°C were kept until they produced their first clutch of eggs (for details see growth experiments). At this point three to six individuals per clonal lineage were placed individually into separate 50 ml plastic falcon tubes containing 50 ml 15°C cold water without any food. The tubes were transferred immediately and simultaneously into a 37°C water bath, in which they stood upright until the measurement was over with spatial randomization over clonal lineages. Starting at 20 min after the tubes had been immersed into the water bath, the tubes were monitored continuously every minute by eye for *Daphnia* activity. T_{imm} was recorded as the time from first placing the tube into the water bath until the time when *Daphnia* did no longer move their thoracopods.

Analysis of fatty acids

For the determination of fatty acid concentrations in *D. magna*, fatty acids from three to four individuals were extracted with 5 ml dichloromethane/methanol (2:1, v:v). Prior to the extraction, 5 μg tricosanoic acid methyl ester (C23:0 ME) in isohexane were added as internal standard. The extraction solvent was evaporated under a stream of nitrogen at 40°C. Then, the evaporated samples were transesterified with 5 ml of 3 N methanolic HCl at 70°C for 20 min to yield fatty acid methyl esters (FAMES) that were extracted with 2 \times 2 ml isohexane. The hexane phase was collected after each of the two isohexane-extraction steps and both extracts were pooled. The pooled extracts were subsequently evaporated to dryness under a stream of nitrogen at 40°C and dissolved in 100 μl isohexane of which 1 μl was subjected to gas chromatographic analysis on a 6890-N GC System (Agilent Technologies, Waldbronn, Germany) equipped with a DB-225 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). The GC conditions were as follows: injector and FID temperatures 220°C; initial oven temperature 60°C for 1 min, followed by a 120°C/min temperature ramp to 180°C, then a ramp of 50°C/min to 200°C followed by

10.5 min at 200°C, followed by a ramp of 120°C/min to 220°C; helium with a flow rate of 1.5 ml/min was used as a carrier gas. A 1 µl aliquot of each sample was injected splitlessly. FAMES were identified by comparing retention times with those of the reference compounds, and then quantified using the internal standard and previously established calibration functions for each individual FAME (von Elert, 2002).

Statistical analysis

Differences among clonal lineages were investigated using one-way ANOVA, if data were homoscedastic. Linear regression analysis was performed, if the equal variance test (for homogeneity of variances) was passed. Analyses were performed with SigmaPlot 11.0.

Results

Time until immobilization (T_{imm}) varied significantly among the 25 *D. magna* clonal lineages isolated from Lake Bysjön, Sweden (one-way ANOVA, $F_{24} = 2.579$, $P = 0.001$, Fig. 1) with mean values ranging from 35 to 50 min.

Subsequently the quantitative fatty acid content was determined in each of the clonal lineages, and the content of total fatty acids (total FAs) and the content of polyunsaturated fatty acids [(PUFAs, the sum of linoleic acid (18:2n-6), α -linolenic acid (18:3n-3), stearidonic acid (18:4n-3), arachidonic acid (20:4n-6), eicosatrienoic acid (20:3n-3), and eicosapentaenoic acid (20:5n-3)] were calculated. The content of total FAs ranged from 46.2 to 103.6 µg/mg dwt (Fig. 2a) and differed significantly among clonal lineages (one-way ANOVA, $F_{29} = 2.717$, $P = 0.001$). The PUFA-content ranged from 27 to 59 µg/mg dry weight (Fig. 2b) and was significantly different among the clonal lineages (one-way ANOVA, $F_{24} = 3.173$, $P < 0.001$). Similarly, the EPA-content differed among clonal lineages (Kruskal–Wallis one-way ANOVA, $H_{25} = 59.193$, $P < 0.001$) with mean values ranging from not detectable (displayed as zero) to 1080 ng/mg dry weight (Fig. 2c).

According to our hypothesis, heat tolerance, here determined as T_{imm} , should be related to membrane fluidity, which itself should be affected by the content of PUFAs in membranes. A linear correlation revealed

that T_{imm} declined significantly with increasing PUFA-content of the different clonal lineages ($r^2 = 0.201$, $P = 0.025$, Fig. 3a). Surprisingly, T_{imm} as well declined significantly with increasing total FAs (data not shown). However, on average PUFAs accounted for 55% of total FAs across all clonal lineages, so that variation in total PUFAs were highly related to variations in total FAs ($r^2 = 0.947$, $P = < 0.001$). It is therefore not surprising that, if total FAs without PUFAs were related to T_{imm} , this relation was no more significant ($r^2 = 0.086$, $P = 0.156$), which indicated that the sum of saturated, mono- and di-unsaturated fatty acids was not related to heat tolerance. In contrast to this, T_{imm} increased significantly with the EPA-content of *Daphnia* clonal lineages ($r^2 = 0.233$, $P = 0.014$, Fig. 3b). T_{imm} was neither significantly related to the content of saturated fatty acids ($r^2 = 0.022$, $P = 0.501$) nor to the content of short-chain mono-unsaturated fatty acids, i.e., the sum of C14:1 n-9 plus C16:1 n-9 ($r^2 = 0.0007$, $P = 0.501898$).

Juvenile somatic growth rates of the *D. magna* clonal lineages at 15°C ranged from 0.12 to 0.21 day⁻¹ and differed significantly among lineages (one-way ANOVA, $F_{25} = 8.69$, $P < 0.001$). Mean juvenile somatic growth rates were not significantly related to the mean PUFA-content of the clonal lineages (linear relationship, $r^2 = 0.001$, $P = 0.884$, Fig. 4a). Similarly, somatic growth at 15°C was not related to the EPA-content of the clonal lineages ($r^2 = 0.021$, $P = 0.495$, Fig. 4b).

Discussion

The potential of populations to adapt to global warming through genotype sorting depends on the degree of variation within the respective population. Here we report significant variation in heat tolerance, measured as knock-out time (time until immobilization, T_{imm}) in a single *D. magna* population that was sampled from May to July 2010. This is the first time that variation in heat tolerance has been assessed in a natural *Daphnia* population. In an earlier paper by Geerts et al. (2015) variation of heat tolerance has been demonstrated within and among experimental populations of *D. magna* that had been kept under different temperature regimes for 2 years, which likely did not fully reflect the initial variation in the

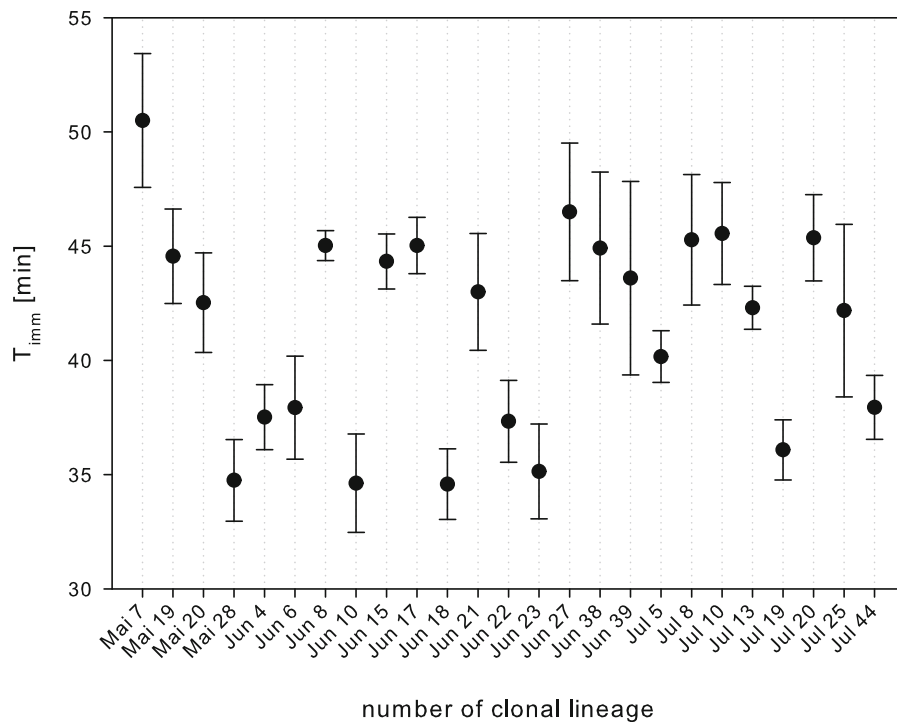


Fig. 1 Mean (\pm SE) time until immobilization (T_{imm}) of 25 *D. magna* clonal lineages isolated from Lake Bysjön grown at 15°C. $N = 3$; except for clonal lineages May 28, Jun 10, Jun 21,

and Jul 19 ($n = 4$); for clonal lineages Jun 6, Jun 27, Jul 20 ($n = 5$); for clonal lineages Jun 22, Jun 38, and Jul 25 ($n = 6$)

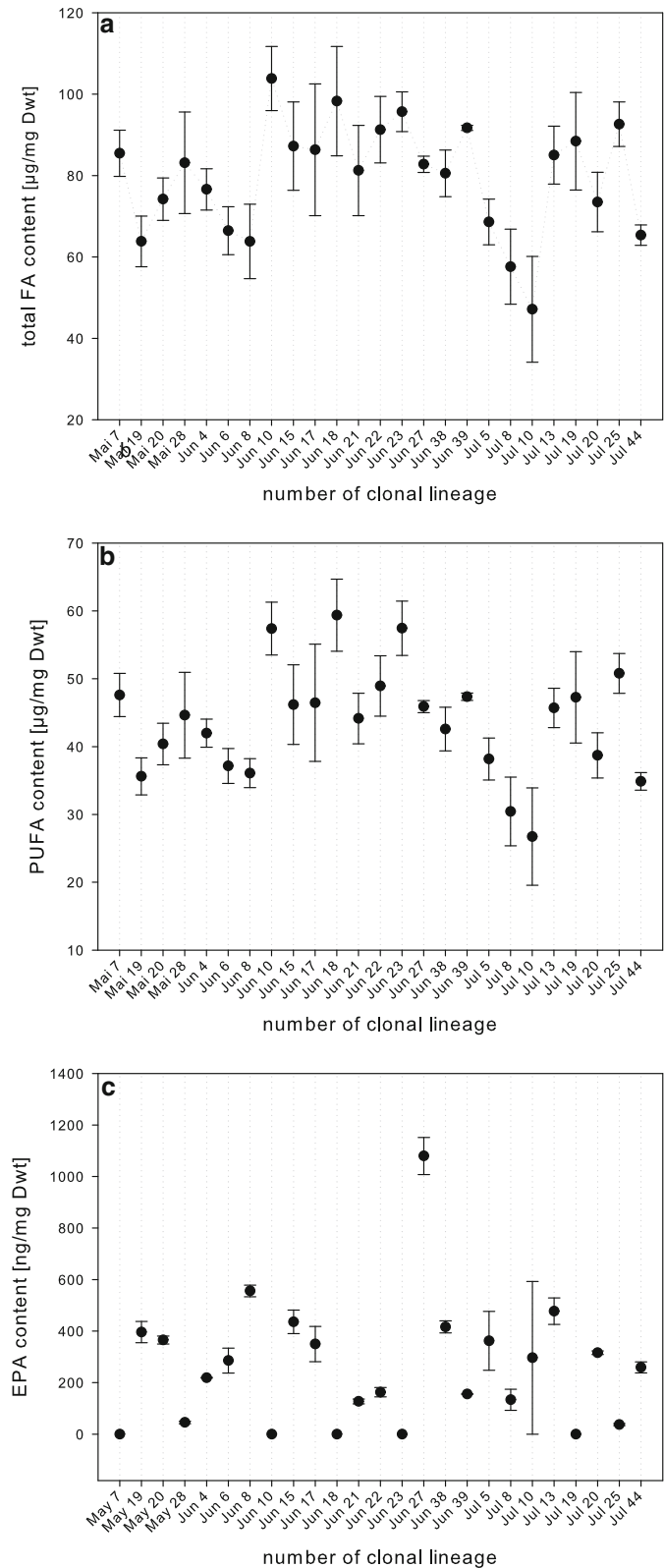
natural population. Geerts et al. (2015) further have shown variation of thermal tolerance among ephippial hatchlings that originated from sediments covering a time period of 10 years. For both the ex-ephippia clones and those from the experimental populations, adaptive changes in heat tolerance were demonstrated over time (Geerts et al., 2015), which indicates that natural *Daphnia* populations harbor sufficient genetic variability to adapt to temperature changes. This adaptation to increased ambient temperatures has recently been linked to changes in gene expression patterns (Jansen et al., 2017). Unfortunately the variation reported in Geerts et al. (2015) is reported in degrees Celsius and can thus not be compared to the variation, which is reported here in units of time, i.e., minutes.

There is increasing evidence that heat tolerance in *Daphnia*, measured as T_{imm} , is related to membrane fluidity. Indirect evidences come from Williams et al. (2012), who demonstrated the existence of a north–south gradient of heat tolerance in North American *D. pulex* and from Yampolsky et al. (2014), who reported that T_{imm} was significantly correlated with average

high temperature at the *D. magna* clones' sites of origin and that T_{imm} strongly increased with the acclimation temperature of the clones. Direct evidence that T_{imm} is a proxy for membrane fluidity of course requires the measurement of membrane fluidity, which rarely has been performed. However, Coggins et al. (2017) have demonstrated that more heat tolerant *D. magna*, in comparison both between acclimation temperatures and among different genotypes, showed lower membrane fluidity than their less heat tolerant counterparts. Based on these findings by Williams et al. (2012), Yampolsky et al. (2014) and Coggins et al. (2017), we did not expect to find variation in T_{imm} as all clonal lineages were established from a single population within a period of 3 months.

The evidences for local adaptation in heat tolerance of *Daphnia* to geographical temperature gradients (Yampolsky et al., 2014; Coggins et al., 2017) are in good agreement with the concept of homeoviscous adaptation according to which poikilotherms try to adjust their membrane lipid composition such that they maintain membrane fluidity (Hazel & Williams, 1990; Hazel, 1995). This adaptation involves

Fig. 2 Mean (\pm SE) content of total fatty acids (total FA content, **a**), of polyunsaturated fatty acids (PUFA-content, **b**) and of eicosapentaenoic acid (EPA-content, **c**) of 25 *D. magna* clonal lineages isolated from Lake Bysjön grown at 15°C. $N = 3$; except for $n = 2$ for clonal lineages May 19, Jun 6, Jun 8, Jun 17, Jun 27, Jun 39, Jul 8, Jul 10, Jul 13, Jul 20



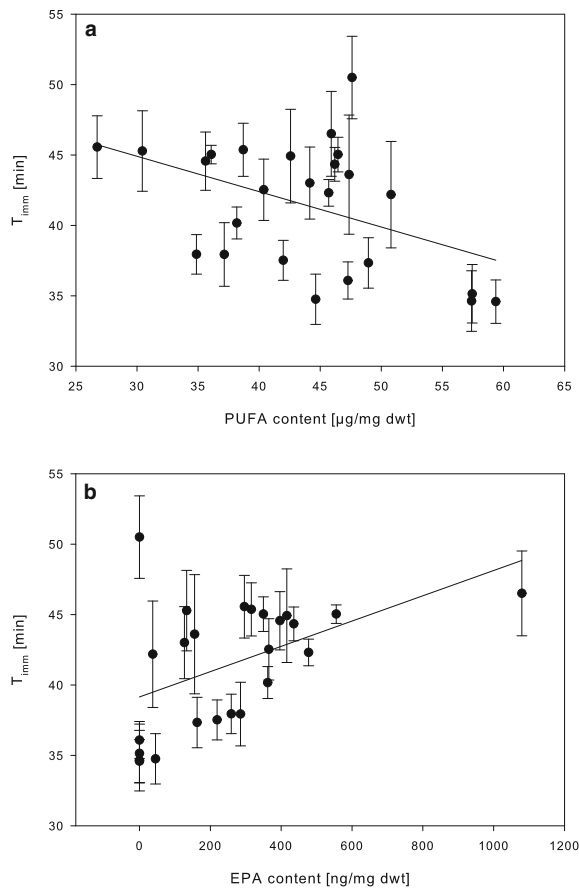


Fig. 3 Time until immobilization (T_{imm}) as a function of **a** mean PUFA-content and **b** mean EPA-content of 25 *D. magna* clonal lineages isolated from Lake Bysjön. Mean (\pm SE), $n = 3$ with exceptions according to Fig. 2. Lines indicate significant linear relations of T_{imm} with PUFA-content and EPA-content of the *D. magna* clonal lineages

remodeling membrane lipids by modifying the chain length and the degree of unsaturation of fatty acids (Guschina & Harwood, 2006). The double bonds in unsaturated fatty acids lead to a more bent carbon chain, which increases the fluidity of membranes containing more unsaturated fatty acids. Thus, poikilotherms are expected to increase the content of polyunsaturated fatty acids (PUFAs) in membranes in response to lower ambient temperatures (Farkas, 1979; Hazel, 1995).

For *Daphnia* there is evidence that body PUFA-content increases with lower ambient temperatures, as concentrations of body-tissue n-3 PUFAs (Sperfeld & Wacker, 2012) and of total PUFAs (von Elert & Fink, 2018) increased at 15°C compared to 20°C. Although

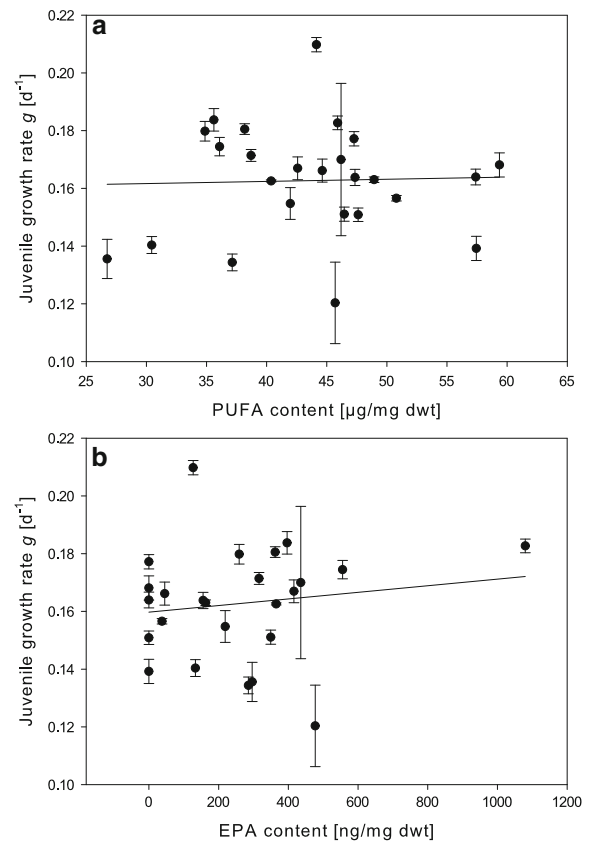


Fig. 4 Mean (\pm SE) juvenile somatic growth rate as a function of **a** mean PUFA-content and **b** mean EPA-content of 25 *D. magna* clonal lineages isolated from Lake Bysjön. $N = 3$; except for clonal lineage Mai 19 ($n = 2$)

the results of these studies are in accordance with the concept of homeoviscous adaptation, these studies did not mechanistically test the concept of homeoviscous adaptation, as neither membrane fluidity nor membrane fatty acids have been analyzed. Sperfeld & Wacker (2011) have demonstrated a higher EPA-requirement at lower temperatures. However, in a series of well designed supplementation experiments Martin-Creuzburg et al. (2012) have nicely demonstrated that at 10°C compared to higher temperatures, *Daphnia* growth may be limited by low dietary provision of PUFAs in general and not by the low availability of a particular PUFA. These findings can be assigned to the more bent carbon chain in double bonds, so that it is not a particular PUFA but rather the overall degree of unsaturation in cell membranes that affects temperature tolerance. This general limitation by PUFAs at lower temperatures reported by Martin-

Creuzburg et al. (2012) provides a strong argument that constraints of *Daphnia* fitness at low temperatures are most probably caused by sub-optimally increased membrane fluidity.

In order to test if the observed variation in T_{imm} might be related to the fatty acid profiles, we here have grown the different clonal lineages at 15°C feeding on *C. reinhardtii*. Since *C. reinhardtii* is a green alga as is *Acutodesmus obliquus* and as α -linolenic acid (C18:3n-3) is the most abundant fatty acid in *A. obliquus* (e.g., von Elert, 2002) and in *C. reinhardtii* (von Elert & Stampfl, 2000; von Elert & Fink, 2018), we expected that, similar to Sperfeld & Wacker (2011) and Sperfeld & Wacker (2012) our experimental conditions would result in PUFA-limited somatic growth. We supposed that, according to Martin-Creuzburg et al. (2012), this EPA-limitation would indicate a general PUFA-limitation, so that the experimental conditions chosen here would result in PUFA-limitation of the clonal lineages. The overall aim was to avoid that excess dietary PUFAs would mask lineage-dependent adjustments in PUFA-content at low temperature. Since we assumed a similar degree of PUFA-limitation in all these lineages, we expected to find similar PUFA-content, which would reflect the minimum content of PUFAs that is needed to build new biomass at 15°C. However, it should be mentioned that here the food alga was grown at 20°C, whereas *Daphnia* were grown at 15°C, and that *Daphnia* growth rates probably would have been affected if *C. reinhardtii* instead had been cultivated as well at 15°C (von Elert & Fink, 2018).

Instead we found mean PUFA values to differ up to twofold among clonal lineages, which suggests that the observed variation reflects clonal differences in assimilation and/or desaturation of fatty acids. We are not aware of a similar analysis within a single *Daphnia* species or even within a single *Daphnia* population.

One might argue, that the unexpectedly high variation in body PUFA-content might be caused by different degrees of PUFA-limitation of the clonal lineages, such that clonal lineages with a high PUFA-content were less PUFA-limited. However, body PUFA-content was not correlated with juvenile growth rate. Hence body PUFA-content may not serve as a proxy for PUFA-limitation, and testing for PUFA-limitation would have required supplementation experiments, which have not been performed. Second, one might argue that we should have analyzed (cell) membrane fatty acids instead of body fatty acids, as

only membrane fatty acid patterns might be modulated according to type and degree of PUFA-limitation, so that the analysis of membrane fatty acids would have resulted in less variability. However, heat tolerance, which may be regarded as a proxy for membrane fluidity (see above), was inversely related to body PUFA-content, which suggests that body PUFA-content and PUFA-content of (cell) membranes are correlated. This inverse relation of heat tolerance and total PUFA-content is in line with an increase of membrane fluidity by PUFAs, as a high PUFA-content of membranes may be detrimental for maintaining vital membrane properties at high temperatures. It is the first time that this relation has been demonstrated by comparison of different clonal lineages from the same population. Still, it remains to be tested, to which degree membrane PUFA-content and body PUFA-content differ, if animals are grown under PUFA-limitation. Third, one might assume that the variability in PUFA-content reflects a genetic variation that becomes visible under PUFA-limited conditions. As the variability in body PUFA-content probably is related to variability in membrane PUFA-content (see above), one might speculate that clonal lineages with a high PUFA-content are genetically adapted to performing stronger diel vertical migration (DVM) than those with a low PUFA-content. De Meester et al. (1995) have documented genetic variation for DVM such that the amplitude of diurnal temperature changes may vary among clones from a single population, and Brzezinski & Elert (2015), using EPA-free food, have shown that the amplitude of DVM increases upon EPA-supplementation. Taken together, these pieces of evidence suggest that clonal lineages with different body PUFA-content would in nature differ in their amplitude of DVM in response to fish and would thus be experiencing different temperature fluctuations although they have shared a common habitat.

This overall inverse relationship between heat resistance and body PUFA-content was not found for EPA. In our experiments EPA accounted, on average, for less than 1% of total PUFAs in *Daphnia* (data not shown), which makes it reasonable to assume that the impact of EPA on membrane fluidity would be rather weak. However, the finding that the relation of heat resistance with body EPA-content was opposite to that observed for total PUFAs points at a unique, yet unknown, function of EPA that differs from that of total PUFAs.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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