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It's about time: Linkages between heat tolerance, thermal acclimation and metabolic rate at different temporal scales in the freshwater amphipod *Gammarus fossarum* Koch, 1836

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ABSTRACT

Temperature has a profound impact on ectotherms. Warming increases the metabolic oxygen demand of ectotherms, which could result in a mismatch between their oxygen demand and their ability to extract and deliver sufficient oxygen to meet demand. This hypothesis has been mainly tested using short-term exposure to intense thermal stress. However, the thermal responses of organisms can be different on longer timescales, where physiological acclimation becomes increasingly important. Such thermal acclimation effects may reduce the vulnerability of ectotherms to warming on the long term. Thus, responses to intense, short-term thermal stress may be different from responses to moderate, prolonged thermal stress. Here, we examine the effect of thermal acclimation on heat tolerance and metabolism in the aquatic ectotherm *Gammarus fossarum* (Koch, 1836). Amphipods were acclimated to either 11.1 ± 0.1 °C or 19.8 ± 0.1 °C and after thermal acclimation we measured both their metabolism and their survival time at different temperatures. Our results show that metabolism strongly increased with increasing temperatures in the cold-acclimated group, but less so in the warm-acclimated group. Cold-acclimated amphipods were also more sensitive to thermal stress, especially during prolonged exposure. Thus, the differences between both thermal acclimation groups support the idea of oxygen-limited heat tolerance: cold-acclimated amphipods showed increased oxygen consumption and decreased thermal tolerance. However, across individuals, those that sharply increased oxygen consumption with increasing temperature did not differ in heat tolerance from individuals whose metabolism was much less sensitive to temperature. Thus, acclimation to different temperatures appeared to be beneficial, but a role for oxygen limitation could not be demonstrated unambiguously. Beneficial effect of acclimation were much larger during prolonged exposure, with the acclimation response ratio (ARR) ranging from 0.03 to over 0.5 depending on the time scale (minutes to months). Thus, the acclimatory capacity may have been underestimated by short-term experimental studies.

1. Introduction

Temperature can be considered a key environmental driver that directly affects physiological processes such as respiration, metabolism, growth, fecundity, thus affecting individual survival, population persistence, biodiversity, and biogeography (Calosi et al., 2010; Cottin et al., 2012). When the temperature rises, energy metabolism and hence oxygen demand of ectotherms also increase. Consequently, there could be a mismatch between the oxygen demand and the ability of organisms to extract and deliver sufficient oxygen to meet the oxygen requirement of their tissue. Such a mismatch and the resulting oxygen limitation is hypothesized to constitute the primary mechanism limiting thermal performance windows (Bozinovic and Pörtner, 2015; Pörtner, 2010). This hypothesis is subject of debate, and strongest evidence for a role of oxygen limitation comes from studies on aquatic ectotherms (Verberk et al., 2016b), although also within aquatic ectotherms there appears variation in the extent to which oxygen is involved (Ern et al., 2015; Koopman et al., 2016; Verberk et al., 2018; Verberk and Bilton, 2013, 2015). Much of the debate centers around the temporal mismatch between the short-term experimental studies used to unravel the physiological mechanisms and the long-term ecological consequences that need to be explained (Kim et al., 2017). A potential complication is that, when organisms are exposed to different temperatures for some time, their thermal responses can be different, reflecting physiological acclimation, which additionally affects the vulnerability of organisms to thermal change (Crickenberger et al., 2015).

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Acclimation to warmer temperatures typically reduces oxygen demand at the higher temperature and thus organisms exhibit a reduced thermal sensitivity, frequently expressed as a Q_{10} value or activation energy (E_a) (Seebacher et al., 2015). At the same time, acclimation to warmer temperatures increases heat tolerance (Calosi et al., 2010; Scharf et al., 2015), although such plasticity in heat tolerance is generally small (Gunderson and Stillman, 2015; Sørensen et al., 2016). Effects of thermal acclimation on heat tolerance and thermal sensitivity of metabolic rate have been rarely linked directly. Magozzi and Calosi (2015) measured both heat tolerance and respiration rates in six caridean prawns from different habitats with contrasting thermal regimes. Their results suggest a link between respiration and heat tolerance as species with a high CT_{max} (critical thermal maximum) also showed high rates of respiration. However, maintaining high rates of respiration and a corresponding high turn-over of energy when faced with short-term, intense heat stress could also be detrimental when faced with prolonged exposure to warming. Verberk and Bilton (2011) measured both respiration rates and CT_{max} in aquatic stonefly nymphs. They found that individuals, which rapidly increased respiration rates in response to warming (high Q_{10} values) displayed reduced maximum critical temperatures, consistent with the idea that oxygen limits heat tolerance. Boardman and Terblanche (2015) found no such relationship between thermal sensitivity of respiration rates (Q_{10} values) and CT_{max}. Jakob et al. (2016) compared 3 species of amphipods from Lake Baikal, and found break point temperatures for ventilation and oxygen consumption to correspond to the onset of mortality. In all these studies, heat tolerance was assessed using the dynamic method, consisting of an exposure trial whereby temperature is ramped up until a critical temperature has been reached, corresponding to some predetermined endpoint (e.g. onset of spasms, loss of coordination or immobility). Methodological differences in ramping rate and starting temperature, which affect the duration of heat tolerance trials, have been shown to affect the resultant heat tolerance (Terblanche et al., 2007).

Rather than a methodological confounding factor, Rezende et al. (2014) argued that a single temperature is insufficient to describe the thermal tolerance of organisms. Organisms can tolerate extremely high temperatures, provided that they are exposed only very briefly, human visits to the sauna being a case in point. Conversely, even minor increases in temperature may have dramatic consequences on the long term, evidenced by species distribution patterns closely matching thermal isotherms, which is also why the current global warming of 2–4 °C is by no means trivial. Thus, thermal stress (or any other stress) has two components: intensity and duration. This idea that stress intensity and duration are both important is not new (Bigelow, 1921). However, explicitly including the effects of thermal stress duration in assays of heat tolerance to describe a tolerance landscape can unveil hidden patterns, such as effects of latitude (Rezende et al., 2014). It may also enable extrapolation of short-term experimental studies to long-term ecological consequences, something which could help resolve the current debate on the role of oxygen limitation and thermal tolerance (Verberk et al., 2016a, 2016b).

In this study, we wanted to test if and how thermal responses in respiration rates map onto the heat tolerance landscape. To this end, we investigated thermal responses in both heat tolerance and respiration rates in amphipods acclimated to two different temperatures. Amphipods (particularly the family Gammaridae) are often considered to be keystone species because of their high abundance, their major role in the processing of organic matter (Hieber and Gessner, 2002; Schmidt, 2003). and their importance as food sources for fish and invertebrate predators, thus being important actors in aquatic food webs (Väinölä et al., 2008; Wallace and Webster, 1996). *Gammarus fossarum* Koch, 1836 is one of the most common freshwater amphipods in European inland streams (Westram et al., 2011). *G. fossarum* is considered to be a sensitive amphipod species towards contamination of water, low oxygen and low pH (Rinderhagen et al., 2000; Verberk et al., 2018). We test whether acclimatory changes in thermal sensitivity of metabolism are related to acclimatory changes in the heat tolerance landscape. Such a tolerance landscape describes the survival time for different intensities of heat stress, thus describing the relationship between intensity and duration of heat stress. We hypothesized that warm acclimated individuals have a lower thermal sensitivity of metabolism and hence lower oxygen consumption rates at high temperatures. If oxygen metabolism is linked to thermal tolerance, with lower oxygen demand enhancing the ability to survive heat stress, this leads to the predication that warm acclimation improves heat tolerance, but that such improvement would be stronger on longer exposure trials, rather than in shorter exposure trials, where protein damage is thought to limit survival more strongly than oxygen deficiency (Kassahn et al., 2009). We would also expect an association between oxygen consumption rates and heat tolerance at the level of individuals, such that heat tolerant individuals have lower Q_{10} values. Such an association between individual metabolism and survival is also hypothesized to be manifested most strongly at the longer exposure trials.

2. Material and methods

2.1. Animal collection and acclimation to different temperatures in lab

Specimens of *Gammarus fossarum* were collected in April 2017 from the Filosofenbeek, a small stream near Nijmegen, the Netherlands (51°49'24.2"N 5°56'33.0"E) and transferred to the lab. Based on the available data on water temperature collected in 2005, the annual average temperature for this stream is around 9.5 °C and during the summer the maximum temperature recorded reached 17.5 °C (Kruijt et al., Unpublished results). During sampling, the temperature of the water was 10 °C. In addition, 200 l of water from the spring was collected and used for maintaining the animals in the lab, and in the experimental set up.

The amphipods were kept in a constant temperature room at 9.9 ± 0.03 °C in a 14 h:10 h L:D regime. In order to prevent cannibalism, the amphipods were sorted into three size categories: large, medium, small. They were fed twice a week with fish food (sera fish food, Germany). To counteract water loss and relative increases in saline concentration by evaporation, the system was regularly topped up with demineralized water. After an initial ten days, amphipods of both large and small size, were separated and acclimated to either 11.1 ± 0.1 °C or 19.8 ± 0.1 °C, 14 h:10 h L:D regime, for at least 7 days to acclimate to the test condition. Amphipods were fed up until 1–2 days before the experiment during the thermal acclimation period to ensure that the amphipods were in a post-absorptive state when their metabolic rate was measured. Mortality during the acclimation was low (< 5%). In total, 5 batches of 20 amphipods were acclimated to either cold and warm conditions, and 76 of them (9 batches of 8 amphipods and 1 batch of 4 amphipods) were used for measuring the respiration rates and heat tolerance. The thermal acclimation system consisted of a flow through set-up whereby the water was either cooled using water chillers (a Grant R5 water bath with a GP200 pump unit, Grant Instrument Ltd., Cambridge, UK) or heated (by means of a Grant TXF200 water bath, Grant Instrument Ltd., Cambridge, UK) before the water flowed by gravity into a common tray which was filtered by a filter pump.

2.2. Respiration measurement

Oxygen consumption was measured for 76 amphipods at 10 °C, 15 °C, 20 °C and 25 °C by using stop-flow respirometry (Table 1). Glass respiration chambers of two sizes (medium: 4 ml and small: 1 ml) were submerged in a temperature controlled aquarium filled with spring water which was continuously pumped through a UV-filter (Sera UV-C-System 5W). Chambers were individually stirred using glass-coated magnetic stirrers (Loligo Systems). Bacterial background respiration was minimized by washing and drying all respiratory chambers with

Table 1
The number of useable measurements and individuals per treatment temperature for both oxygen consumption and heat tolerance measurements.

Number of measurement for each trials	Test temperature	Acclimation temperature (11.1 °C)	Acclimation temperature (19.8 °C)	Number of individuals for both acclimation group
# $\dot{M}O_2$ measurements	10 °C	65	68	76
# $\dot{M}O_2$ measurements	15 °C	68	65	76
# $\dot{M}O_2$ measurements	20 °C	70	64	76
# $\dot{M}O_2$ measurements	25 °C	76	67	76
# Heat tolerance measurements	30 °C	9	9	18
# Heat tolerance measurements	31 °C	12	9	21
# Heat tolerance measurements	32 °C	9	9	18
# Heat tolerance measurements	33.5 °C	10	9	19

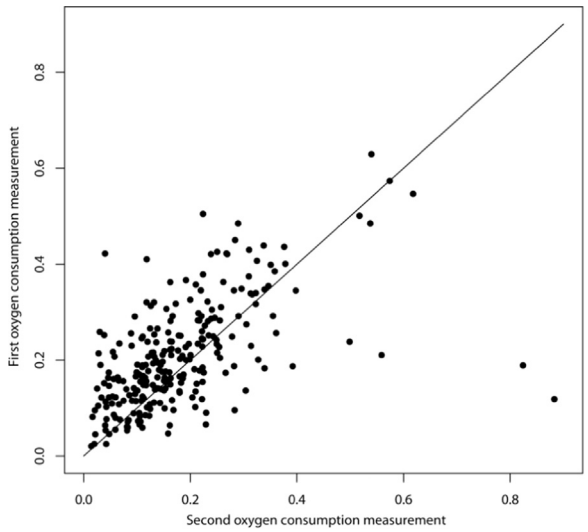

Fig. 1. Relationship between the first respiration measurement and the second respiration measurement. Line indicates y = x.

ethanol and demi water prior to each experiment. Respiration chambers were fitted with a metal mesh to form a false bottom to prevent contact between the amphipod and the magnetic stirrer bar. Individuals were allowed to acclimate for 30 min before the chambers were closed. Each chamber was fitted with an oxygen sensor spot (2 mm in diameter, PreSens, Precision Sensing GmbH). Oxygen concentrations were then measured every 15 s using a 10-channel Fiber-Optic Oxygen Meter (OXY-10, PreSens, Precision Sensing GmbH) for 30 min. Eight chambers with amphipods and two blanks were measured in parallel. A peristaltic pump (Gilson, minipuls 3, Gilson International B.V.) then flushed the chambers for 15 min, after which oxygen concentrations were measured once more for 30 min. After two such measurements (Fig. 1), the peristaltic pump flushed the chambers continuously and the temperature was increased to the next measurement temperature, after which the procedure was repeated. Oxygen concentrations in each chamber were logged and from the decrease of the oxygen in the last 15 min of a measurement cycle (expressed as the slope fitted by a linear regression in μmol min^{-1} L^{-1}). The respiration rate was calculated with the formula below:

$$MO_2\ [\mu mol\ h^{-1}\ Ind^{-1}] = (respiration\ slope - control\ slope) \times 60 \times \frac{volume\ (ml)}{1000}$$

We calculated the volume of each chamber gravimetrically using the following formula:

$$volume = (full\ chamber(water + animal)) - (empty\ chamber) - (fresh\ weight\ of\ anmial(g))$$

Temporal variations in oxygen concentrations in blanks were characterized by low respiration rate (− 1%). Slope estimates from linear regressions that yielded poor model fits for temporal variations in oxygen concentrations in chambers with amphipods ($R^2 < 0.5$) were discarded from the analysis. In this way, 608 measurements made for 4 temperatures on 76 individuals yielded 543 usable respiration rates.

2.3. Q_{10} of respiration

The Q_{10}-factor by which the oxygen consumption rate increases when the temperature is raised by about ten degrees was measured at six temperature intervals (10–15, 10–20, 10–25, 15–20, 15–25 and 20–25 °C). Based on the equation below, R1 is the measured respiration rate at temperature T1, and R2 is the measured respiration rate at temperature T2.

$$Q_{10} = \left(\frac{R2}{R1}\right)^{\left(\frac{10}{T2-T1}\right)}$$

2.4. Heat tolerance measurement

Survival was always assessed on the day following the respiration measurements. One of four test temperatures were employed: 30 °C, 31 °C, 32 °C, and 33.5 °C. Given the lethality of these assays and in contrast to respiration measurements, each amphipod was only assessed at one temperature (Table 1). Amphipods were placed in glass petri dishes placed upside down on a semi-transparent nylon mesh, so that water was able to flow through the chamber freely, without allowing the amphipods to escape. The amphipods were placed in a temperature-controlled tray (using a Grant Industries TXF200 system) filled with spring water. An UV-filter mechanism (Sera UV-C-System 5W) was active to cleanse the water continuously and the water was aerated to ensure that the water was air-saturated. The survival time of amphipods were recorded by using a video recording system (ThorLabs APT ThorCam software version 2.6.7064, ThorLabs Inc. 1.4 Mpixel CCD camera) to allow for more accurate estimation of the time of death. This was particularly necessary for the measurements at relatively low temperatures, because the occasional long survival times (> 8 h) meant that amphipods would die outside office hours. Whenever possible, the amphipods were also observed in person, to verify the time of death estimated from the videos. Given the large difference between the acclimation temperatures (11.1 ± 0.1 °C or 19.8 ± 0.1 °C) and the test temperature (> 30 °C) we first exposed all individuals after transferal from their thermal acclimation conditions to an aerated aquarium with a water temperature of 25 °C for 5 min to minimize any shock of exposure to the higher test temperatures. Every trial included 8 individuals (consist of 4 small and 4 large) from a given acclimation temperature (11.1 ± 0.1 °C; 19.8 ± 0.1 °C) (Table 1 for exact numbers). An amphipod was considered dead if it had not shown any movement for 2 min. For these individuals, the last time movement was taken as the time of death. After the experiment, the amphipods were blotted

dry before determining their fresh weight. Then they were stored in 70% alcohol for identification to ascertain that all individuals used were indeed *G. fossarum*. The sex of the individuals was also noted during identification. After identification, individuals were dried to a constant weight (3 days at 70 °C) and their dry weight was measured.

2.5. Activation energy (E_a) of respiration and survival

The thermal dependency of survival time, expressed as the activation energy E_a was calculated for both acclimation groups using the following equation (Castañeda et al., 2015):

$$E_a = \frac{2.303 R T_{min} T_{max}}{z}$$

where E_a is expressed in J mol^{-1}, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), and T_{min} and T_{max} are the minimum and maximum test temperatures and z is defined by the slope of the curve describing how thermal tolerance decays with the duration of the thermal challenge (see Rezende et al., 2014).

We also calculated the thermal dependency of metabolism, expressed as the activation energy E_a using the Arrhenius equation:

$$Ea = -R \times S$$

where E_a is expressed in J mol^{-1}, R is the gas constant (8.314 J mol^{-1} K^{-1}), and S is the slope of the Arrhenius plot.

2.6. Acclimation response ratio (ARR)

We determined the acclimation response ratio (ARR) which is a method to compare the thermal acclimation ability of an organism. It is a ratio between the difference of the survival temperatures (ST), for cold and warm acclimated groups, divided by the difference between the acclimation temperatures:

$$ARR = \frac{\Delta ST}{\Delta \text{acclimation temperature}}$$

The ARR was calculated for different durations of heat stress (ranging from 1 min to 1 year). To do so we calculated the survival temperature (ST) for a given duration and a given thermal acclimation group. The difference in ST between the warm acclimated amphipods and the cold acclimated amphipods gives ΔST. ST's were inferred from our results that describe the linear relationship between stress duration (i.e. the logarithm of survival time) and heat stress intensity (i.e. the test temperature) in the two thermal acclimation groups.

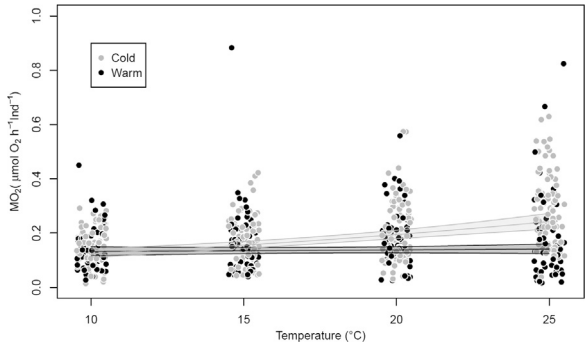

Fig. 2. Oxygen consumption rates (log scale) at different test temperatures in cold (grey circle) and warm (black circle) acclimated amphipods.

2.7. Data analysis

In our analysis of the respiration data, the logarithm of oxygen consumption ($\dot{M}O_2$) was considered as the dependent variable, the logarithm of mass (fresh or dry), acclimation temperatures and test temperatures were used as independent variables. Respiration of the same individual from cold and warm acclimated groups was measured twice at four different temperatures. Preliminary analyses demonstrated that these two respiration measurements were correlated (Fig. 1; Spearman rank, r = 0.619; $P < 0.0001$), indicating consistent individual differences. The second measurement was broadly similar to the first one, but on average slightly lower (t-value = − 4.802853, $P < 0.0001$). Both measurements were included in the model and we obtained a maximum of 8 measurements for a given individual. To account for the non-independence of repeated measures from the same individual, we fitted a linear mixed effects model lme() from the "nlme" R-package (Pinheiro et al., 2012), with individual as a random factor (Pinheiro and Bates, 2000). We also included sequence (first or second measurement) in the model.

In our analysis of the survival data, the (log transformed) survival time was included as the response variable, logarithm of mass (fresh or dry), acclimation temperatures and test temperatures were used as explanatory variables in the model. Preliminary analyses showed that there was no significant effect of sex on survival and respiration. We used the function {testInteractions} from the "phia" R-package to calculate the significance of the contrast between both thermal acclimation groups for various test temperatures.

To test our hypothesis that the thermal sensitivity of the metabolism of an individual was related to its heat tolerance, we performed two analyses. First, we averaged the Q_{10} value for thermal responses in metabolic rate across the 6 temperature intervals and tested how well these could predict the residuals of the model for heat survival mentioned above. Average Q_{10} values greater than 5 were omitted from this analysis. Secondly, we directly included this average Q_{10}-value as a predictor in the survival model. All analyses were performed in RStudio Version 1.0.136 with standard packages and analysis were considered significant with a p value ≤ 0.05.

3. Results

3.1. Respiration

Oxygen consumption rates increased with test temperature and this thermal response differed between the cold and warm acclimated groups (temp × thermal acclimation: t-value = − 5.04, $P < 0.0001$; Fig. 2; Table 2). Amphipods had somewhat similar mass and differences in mass did not strongly affect respiration rates: only a small effect was

Table 2
Result of mixed effects regression model on differences in oxygen consumption (log-transformed) as a function of test temperature, thermal acclimation, fresh weight, and sequence (first or second measurement).

Factor	Estimate	DF (num,den)	t-value	p- value
Intercept	− 0.5397471	1,464	− 3. 099	0.0021
Temperature	0.0196275	1,464	7.839	< 0.0001
Acclimation warm	0.2142919	1,73	2.914	0.0047
Sequence	− 0.0962073	1,464	− 4.803	< 0.0001
Log fresh weight	0.2531348	1,73	2.557	0.0126
Temp: acclimationwarm	− 0.0179873	1,464	− 5.043	< 0.0001

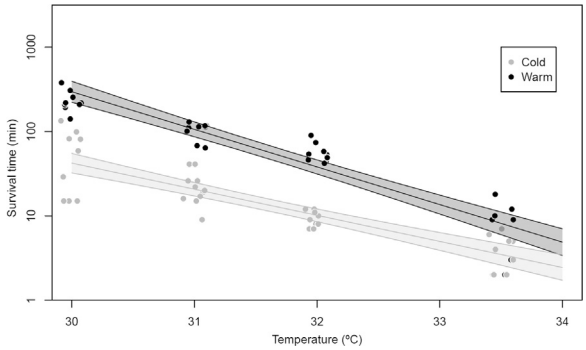

Fig. 3. Heat tolerance of cold (grey circle) and warm (black circle) acclimated amphipods tested at four different stress temperatures.

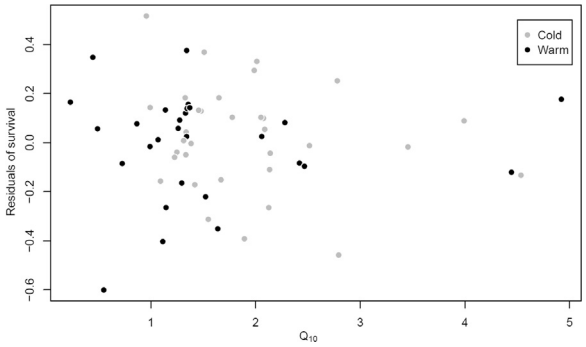

Fig. 4. Plot of the residual survival time of an individual against its mean Q_{10} value for metabolism. Cold-acclimated individuals are shown in grey and warm-acclimated individuals are shown in black.

Table 3
Result of linear regression model on differences in survival time (log-transformed) as a function of stress temperature, thermal acclimation, and dry weight.

Factor	Estimate	DF (num,den)	*t*-value	*p*-value
Intercept	11.51463	1,71	12.048	< 0.0001
Temperature	− 0.30950	1,71	− 10.999	< 0.0001
Acclimation warm	4.94007	1,71	3.853	0.00025
Log dry weight	0.24137	1,71	1.987	0.05082
Stress.temp: acclimationwarm	− 0.13644	1,71	− 3.368	0.00123

found when mass was expressed as fresh weight (*t*-value = 2.55, $P = 0.012$), but when expressed as dry weight the effect was not significant (*t*-value = 1.41, $P = 0.16$). A random effects model that included individual as a random factor and acclimation temperature, test temperature and their interaction and sequence (first or second measurement) could explain 39% of the variation (conditional R^2), with 20.9% being related individual differences captured by the random effect and 18.1% (marginal R^2) to fixed effects.

3.2. Survival

In both cold and warm acclimated groups, survival was strongly affected by test temperature (test temperature: *t*-value = − 10.999; $P < 0.0001$; Fig. 3; Table 3) with survival time declining with more intense heat stress (Fig. 3; Table 3). The heat tolerance landscape of the warm-acclimated group differed from that of the cold acclimated, as both the intercept (*t*-value = 3.853, $P = 0.00025$) and slope (test temperature × thermal acclimation: *t*-value = − 3. 368, $P = 0.00123$) of the thermal death curve differed with thermal acclimation (Table 3). Consequently, when compared to cold-acclimated amphipods, warm-acclimated amphipods showed enhanced tolerance, especially under longer heat stress at the lowest test temperature, but their survival time decreased faster with increasing temperatures, such that survival time became statistically indistinguishable at 34.5 °C (F-value = 3.2827; $P = 0.0742$). We also found a tendency for large individuals to be more heat tolerant when weight was expressed as dry weight (*t*-value = 1.987; $P = 0.0508$), but no such effect when weight was expressed as fresh weight (fresh weight: *t*-value = 1.29; $P = 0.201$).

3.3. Thermal sensitivity of respiration rate and survival across thermal acclimation groups

The mean Q_{10} values (averaged across the 6 temperature intervals) for cold-acclimated individuals was higher (1.92) than that for warm-acclimated individuals (and 1.47). To test our hypothesis that the thermal sensitivity of the metabolism of an individual was related to its heat tolerance we regressed this mean Q_{10} value for metabolic rate against the residuals of the model for heat survival (Fig. 4). No significant correlation was found between the thermal sensitivity of the metabolism of an individual (expressed by the mean Q_{10} value) and the residuals of the model for heat survival (*t*-value = − 0.226; $P = 0.822$). Also, when directly included in the survival model, an individual's mean Q_{10} values was not significantly related to heat tolerance (*t*-value = − 0.481; $P = 0.632$).

Table 4
Activation energy (E_a) of respiration and survival time for cold and warm acclimated groups.

Acclimated groups	E_a of respiration (kJ mol^{-1})	E_a of survival time (kJ mol^{-1})
Cold-acclimated	31.49	557.08
Warm-acclimated	2.88	811.26

3.4. Activation energy (E_a) of respiration and survival

The activation energy of respiration for the cold acclimated group is higher than that for the warm acclimated group, reflecting the stronger increase in respiration rates with temperature (Table 4). In contrast, the activation energy for survival was higher in warm acclimated individuals than in cold acclimated ones, reflecting the stronger decline in survival time of warm acclimated individuals with increasing temperatures.

3.5. Acclimation response ratio (ARR)

The acclimation response ratio (ARR) was calculated as a measure of the extent to which thermal acclimation of *Gammarus fossarum* changed heat tolerance. Since the thermal death curves diverge (difference in slopes; see Fig. 3), the ARR differs with the duration and hence intensity of heat stress, ranging from 0.03 on a time scale of 1 min to 0.68 on a time scale of 1 year (Table 5).

Table 5
The Acclimation Response Ratio (ARR) for the values of survival temperature.

	1 min	1 h	1 day	1 week	1 month	1 year
ST (11.1 °C acclimation)	35.21	29.47	25.01	22.28	20.22	16.73
ST (19.8 °C acclimation)	35.51	31.53	28.43	26.54	25.11	22.69
ARR	0.03	0.23	0.39	0.49	0.56	0.68

4. Discussion

In the face of global warming, both the overall level of tolerance against thermal extremes and the ability to shift these with acclimation are considered fundamentally important (Gunderson and Stillman, 2015; Donelson and Munday, 2012; Huey et al., 2012; Somero, 2010; Stillman, 2003). During the process of acclimation, a variety of physiological responses, including the expression of new proteins (such as heat-shock proteins or isozymes) and remodeling of cell membranes leading to changes in fluidity and permeability can lead to adjustments in the energy requirement of an individual leaving it better equipped to deal with the new thermal conditions (Angilletta, 2009). In this study, we tested whether acclimation to different temperatures influenced thermal responses in oxygen consumption and whether this physiological remodeling could explain shifts in heat tolerance.

Seebacher et al. (2015) showed that thermal acclimation decreases the thermal sensitivity of respiration in freshwater and marine animals to the temperature changes. Our results demonstrate similar differences in thermal sensitivity between thermal acclimation groups: warm-acclimated individuals displayed lower respiration rates than cold-acclimated individuals, especially at warmer test temperatures (Fig. 2). Moreover, the mean Q_{10} values (1.92–1.47) are within the range reported for crustaceans in the literature (Cumillaf et al., 2016; Daoud et al., 2007).

We also found support for our hypothesis that thermal acclimation improved heat tolerance. Warm-acclimated amphipods could tolerate heat stress for longer. In both thermal acclimation groups, we found the expected decrease in survival time when heat stress become more intense (Rezende et al., 2014). However, the difference between both thermal acclimation groups differed along a heat stress intensity and heat stress duration gradient, becoming more pronounced during prolonged exposure to relatively moderate heat stress. As survival time was expressed on a log scale, the divergence between both thermal acclimation groups is more than proportional. Castañeda et al. (2015) have found similar differences with acclimation temperature in the heat tolerance landscapes of *Drosophila subobscura* Collin, 1936. Thus, not only can the duration affect the outcome of assays of heat tolerance (Terblanche et al., 2007), but explicitly including time in these assays also gives a more complete picture of the effect of thermal acclimation. As pointed out by Sørensen et al. (2016), a focus on extreme thermal tolerance gives an incomplete picture. To illustrate this, we calculated the ARR and show that depending on the time of exposure to heat stress we can find values ranging between 0.03 (1 min) and 0.68 (1 year), which in fact span the whole range of ARR values reported (Gunderson and Stillman, 2015). Gunderson and Stillman (2015) conclude that thermal acclimation is insufficient to buffer animals from the effects of global warming. However, when extrapolating these short-term assays to long-term consequences of more ecological realism (characterized by longer exposure to heat stress of relatively low intensity), it becomes clear that thermal acclimation may have substantially larger effects than previously considered. True, even for long survival times of 1 year ARR values do not approach the value of 1 where thermal acclimation is complete. However, in our study, we only acclimated individuals for a week and longer acclimation times and transgenerational effects may result in even larger differences (Morley et al., 2017). Differences in thermal physiology may be further enlarged by (local) adaptation. For example, Foucreau et al. (2014) compared southern, warm adapted populations and northern, cold adapted populations of *Gammarus pulex* Linnaeus, 1758, demonstrating that southern populations were more heat tolerant.

While thermal acclimation affected both thermal sensitivity of oxygen consumption and thermal sensitivity of heat tolerance, we did not find any evidence for a direct link (Fig. 4), counter to our expectation. Our calculation for the activation energy of oxygen consumption showed a higher *Ea* (31.49 kJ mol^{-1}) in the cold-acclimated group than in the warm-acclimated group where *Ea* was lower (2.88 kJ mol^{-1}), as evidenced by the greater increase in oxygen consumption with increasing temperature. Clearly, the cold-acclimated group expend more energy and consume more oxygen at higher temperatures. So, across both thermal acclimation groups there appears to be a link with cold-acclimated amphipods requiring more oxygen at the higher temperatures and displaying reduced heat tolerance. Similarly, Maazouzi et al. (2011) compared the amphipods *Dikerogammarus villosus* Sowinsky, 1894 and *Gammarus pulex* and found that *Dikerogammarus villosus* both had a stronger increase in oxygen consumption with temperature (higher thermal sensitivity in metabolism) and a reduced heat tolerance. However, if oxygen limitation sets heat tolerance, one would predict that the reduction in heat tolerance in the cold-acclimated group is especially pronounced at higher temperatures, since differences in metabolism were greatest at the highest test temperature of 25 °C (Fig. 2). While the cold-acclimated group indeed displayed lower heat tolerance in general, the difference was smaller when tested at higher temperatures, opposite to the expectation based on greater differences in oxygen consumption at higher temperatures. This is also reflected in the fact that the *Ea* for survival time is higher in the warm-acclimated group (811.26 kJ mol^{-1}) than in the cold-acclimated group (557.08 kJ mol^{-1}). These differences in *Ea* are similar in magnitude and direction as those reported by Castañeda et al. (2015) for warm reared flies (*Ea* = 438.7 kJ mol^{-1}) and cold reared flies (*Ea* = 387.0 kJ mol^{-1}). The small differences in heat tolerance observed at the higher test temperatures where differences in oxygen consumption are greatest lends support to the interpretation that, at these higher temperatures, heat tolerance is disconnected to oxygen metabolism. Instead, animals switch to anaerobic metabolism and activate cellular stress-responses allowing passive, time-limited survival and these responses may exhibit less plasticity following thermal acclimation (e.g. Kassahn et al., 2009). At lower temperatures, the role of oxygen metabolism in setting heat tolerance may be larger, acting not only via increased mortality induced by oxygen limitation, but likely also via reductions in growth and reproduction (Verberk et al., 2016a).

At the level of the individuals, we did not see an association between heat tolerance and oxygen consumption: individuals, which displayed a high thermal sensitivity in their oxygen consumption rates, did not display reduced heat tolerance. Such a relationship was also absent for the lower test temperatures, where oxygen metabolism may play a larger role in setting heat tolerance (see above). Since our oxygen consumption data were somewhat noisy (conditional R^2 = 39%), we cannot discount the possibility that there was a relationship between metabolism and heat tolerance, but that it went undetected. Another possibility is that under normoxia, individuals of *G. fossarum* did not run out of oxygen and thermal tolerance was limited by other mechanisms such as neuronal dysfunction (Ern et al., 2015) or protein inactivation (Van der Have, 2002). These mechanisms likely become more important in setting tolerance limits when heat stress becomes more intense, which could explain the smaller differences between the acclimation groups when tested at the higher stress temperatures as noted above. It is also possible that individuals died from starvation, not lack of oxygen, although the species has been shown to tolerate starvation for up to 28 days in water of 11 ± 0.4 °C (Hervant et al., 1999). Verberk et al. (2018) report an increase in CTmax in hyperoxic water and a decrease in hypoxic water for *G. fossarum*. To tolerate the prolonged exposure to heat in our trials, individuals will have had to sustain oxygen supply for longer time periods (especially at the lowest test temperature of 30 °C) and it is possible that this capacity is not reflected by our measured rates of oxygen consumption and critical PO_2 tests are potentially more relevant here (see Verberk et al., 2018).

In conclusion, our study significantly advances our understanding regarding the effect of thermal acclimation on thermal responses in oxygen consumption rate of amphipods and their sensitivity to heat stress. Our results demonstrate that acclimation to different temperatures appeared to be beneficial, especially at longer timescales. While thermal acclimation had an effect on both respiration and heat

tolerance, we did not find evidence for a direct link. This suggests that our measurements of oxygen consumption rates may not have reflected capacity limitations to supply oxygen, or that such capacity limitations were not involved in limiting heat tolerance. The beneficial effect of acclimation was much larger during prolonged exposure and the acclimatory capacity of species may have been underestimated by short-term experimental studies.

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Declarations of interest

None.

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