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Changes in heat stress tolerance in a freshwater amphipod following starvation: The role of oxygen availability, metabolic rate, heat shock proteins and energy reserves

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ABSTRACT

The ability of organisms to cope with environmental stressors depends on the duration and intensity of the stressor, as well as the type of stress. For aquatic organisms, oxygen limitation has been implicated in limiting heat tolerance. Here we examine how starvation affects heat tolerance in the amphipod *Gammarus fossarum* (Koch, 1836) and whether observed changes can be explained from alterations in oxidative metabolism, depletion of energy reserves, upregulation of heat shock proteins or susceptibility to oxygen limitation. Starved amphipods showed impaired survival compared to fed amphipods during prolonged exposure to mild heat. In contrast, under acute, high-intensity heat exposure they actually showed improved survival. We observed a lower demand for oxygen in starved amphipods which could make them less susceptible to oxygen limitation. Such a role for oxygen in limiting heat tolerance was verified as hypoxia impaired the heat tolerance of amphipods, especially starved ones. Fed amphipods likely rely more on anaerobic metabolism to maintain energy status during heat stress, whereas for starved amphipods aerobic metabolism appears to be more important. The depletion of their energy reserves constrains their ability to maintain energy status via anaerobic metabolism. We did not find evidence that alterations in heat tolerance following starvation were related to the upregulation of heat shock proteins. In conclusion, starvation can have opposite effects on heat tolerance, acting via pathways that are operating on different time scales.

1. Introduction

Environmental stressors such as high temperature, low oxygen and food limitation impact the fitness of organisms by progressively reducing their reproduction, growth, activity, and ultimately survival (Cheng et al., 2018; Chidawanyika et al., 2017; Colinet et al., 2010; Fitzgibbon et al., 2017). Some stressors appear to be linked with cellular processes that protect against one stressor also protecting against another stressor such as cold stress and desiccation (Sinclair et al., 2013). Given the low availability of oxygen in water (Dejours, 1981; Verberk et al., 2011), temperature driven increases in oxygen demand may cause aquatic ectotherms to run out of oxygen and hence energy in warm waters. As a result, heat stress and oxygen deficiency appear to be linked in aquatic organisms (Pörtner, 2010; Verberk and Bilton, 2013), although the strength and nature of this linkage is debated (Jutfelt et al., 2018; Verberk et al., 2016). To maintain energy status, organisms may switch to anaerobic metabolism, which frequently involves upregulation of pathways to increase glucose levels and maintain glycolysis (Malmendal et al., 2006; Verberk et al., 2013). This makes energy reserves an important factor in surviving heat stress, especially on longer temporal scales. Most organisms face starvation stress during their lifetime, which refers to the condition when an animal is unable to consume enough food for covering its minimum energetic requirements (Mir and Qamar, 2018; Scharf et al., 2016). Hence, understanding how ectotherms deal with a combination of starvation and heat stress will help predict their responses to a warmer future.

Starvation and heat tolerance can be linked via several mechanisms (Fig. 1). When facing starvation, animals may conserve ATP by down-regulating their metabolism to a hypo-metabolic state. In this state, protein turnover and ATP supply pathways are reduced, which is reflected by a lower rate of oxygen consumption (Hervant, 2012; McCue, 2010). If the tolerance to heat of an individual arises from a mismatch

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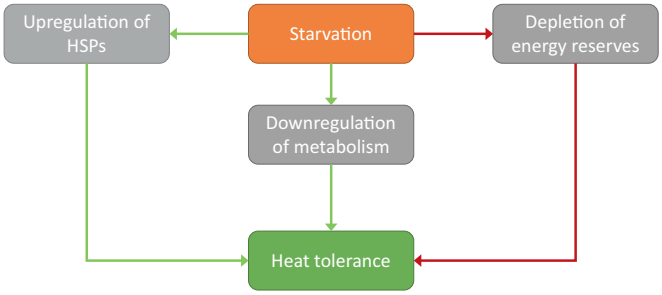

Fig. 1. Schematic illustration of several mechanisms influencing the link between starvation and heat tolerance. Green pathways: increase heat tolerance, Red pathway: decrease heat tolerance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between oxygen supply and its demand for oxygen, a reduction of oxygen demand in starved individuals could improve their heat tolerance (Fig. 1). A cellular response to starvation is the production of heat shock proteins (Cara et al., 2005; King and MacRae, 2015; Yengkokpam et al., 2008). Heat shock proteins act as molecular chaperones which help to maintain protein function in different ways, ranging from the prevention of protein denaturation or inactivation, to repair and degradation of damaged proteins (Feder and Hofmann, 1999). The upregulation of heat shock proteins occurs in response to a range of stressors, including desiccation, starvation, cold, but also heat, as their name suggests (King and MacRae, 2015). Therefore, upregulation of heat shock proteins, due to starvation, may result in cross tolerance to other stressors such as heat (Sinclair et al., 2013; Nguyen et al., 2017). Finally, starvation and heat tolerance may be linked energetically (Fig. 1). Lipids are the most important energy reserve an organism can use under starvation, with animals being able to survive starvation for longer if they have more fat reserves (Scharf et al., 2016). Glycogen is another energy reserve that is used under starving conditions. Severe, prolonged starvation can result in muscle catabolism serving as a source of energy and protein (McCue, 2010) thereby allowing animals to maintain homeostasis and provide fuel for vital metabolic pathways (Hervant, 2012). Given the importance of energy reserves during heat tolerance (Malmendal et al., 2006; Verberk et al., 2013), starvation may lead to a reduction in heat tolerance (Manenti et al., 2018). Thus, the effect of starvation on heat tolerance can be either positive or negative depending on the mechanism involved (Fig. 1).

Previous studies regarding starvation effects on heat tolerance have shown inconsistent results (Table 1), with some studies demonstrating that starvation improved heat tolerance (DeVries et al., 2016; Gotcha et al., 2018; Mutamiswa et al., 2018), whereas others found heat tolerance to be impaired (Mir and Qamar, 2018; Nyamukondiwa and Terblanche, 2009). While these variable outcomes could partly reflect differences in methodology across studies (e.g. static or dynamic methods to measure heat tolerance), such differences could also arise because different mechanisms (see Fig. 1) may act in tandem, but on different timescales. For example, the depletion of energy stores occurs in the time course of weeks, whereas the induction of heat shock proteins occurs within hours. In addition, the ability to tolerate a stressor such as heat depends not only on the intensity of stress but also the exposure duration (Rezende et al., 2014) and both these aspects of stress can be modulated by physiological acclimation (Semsar-kazerouni and Verberk, 2018).

In this study, we used *Gammarus fossarum*, a species which is highly sensitive to both warming and low oxygen (Verberk et al., 2018). This group of amphipods is frequently used as a bioindicator for water quality and has an important role in the food web of freshwater ecosystems (Rinderhagen et al., 2000). Here we studied the effects of starvation on heat tolerance of amphipods. We also compared levels of heat shock protein, metabolic rate and energy reserves between starved and fed amphipods to evaluate the different mechanisms proposed (Fig. 1). To evaluate the role of oxygen and oxygen-based mechanisms we also studied the effects of hypoxia on the heat tolerance of amphipods and whether this was different between fed and starved animals. Since the temporal scale on which these mechanisms act might differ, we explicitly included time as a factor in our measurements of heat tolerance.

2. Materials & methods

2.1. Animal collection and acclimation conditions

Gammarus fossarum (Koch, 1836) were collected on July 16th 2018 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. During sampling, the temperature of the water was 15.5 °C. Based on the available data of the 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 D.B., R.S.E.W. Leuven & G. van der Velde, unpublished data). In addition, 150 L of spring water was collected and used for housing the animals in the lab and in the experimental setup. After the collection of amphipods, they were maintained in a climate chamber under a constant temperature of 10 °C with a 16 h:8 h L:D regime for at least one week prior to the start of the experiment to allow the animals to adjust from the effects of capture and transfer to laboratory conditions. Only after this initial period did we start with the experiment. Since we were limited in how many animals we could process for the subsequent measurements of the various response variables (metabolism, heat tolerance) we started allocating amphipods to either the fed or starved group on multiple time

Table 1
Overview of studies that have investigated the effects of starvation on heat tolerance in ectotherms.

Species	Effect of starvation on heat tolerance	Duration of trial to establish level of heat tolerance	Reference
Fruit fly (*Drosophila melanogaster*)	Negative correlation between heat tolerance and period of starvation	Between 80 and 1200 min	Manenti et al., 2018
Beetle (*Zygogramma bicolorata*)	Reduction of heat tolerance	~ 80 min	Chidawanyika et al., 2017
Fruit fly (*Ceratitis capitata / Ceratitis rosa*)	Reduction of heat tolerance	~ 70 min	Nyamukondiwa and Terblanche, 2009
Ant (*Aphaenogaster picea*)	Reduction of heat tolerance	~ 40 min	Nguyen et al., 2017
Silkworm (*Bombyx mori*)	Reduction of heat tolerance	~ 7.8 min	Mir and Qamar, 2018
Stemborer (*Chilo partellus*, *Busseola fusca* and *Sesamia calamistis*)	An improvement in heat tolerance in Chilo partellus	~ 84 min	Mutamiswa et al., 2018
Fruit fly (*Ceratitis rosa*)	An improvement in heat tolerance	~ 72 min	Gotcha et al., 2018
Bed bugs (*Cimex lectularius*)	An improvement in heat tolerance	~ 50 min	DeVries et al., 2016
Fruit fly (*Ceratitis capitate*)	An improvement in heat tolerance	~ 8.5 min	Mitchell et al., 2017
Flour beetle (*Tribolium castaneum*)	No effect on heat tolerance	~ 7 min	Scharf et al., 2016

points. As a result, at the time amphipods were measured, the time after capture ranged between 1 and 7 weeks and averaged at 4 weeks. In order to prevent cannibalism, the amphipods were sorted into three size categories: large (> 10 mm), medium (± 10 mm), small (< 10 mm). They were fed three times a week with fish food (sera fish food, Germany). To compensate evaporation, the system was regularly topped up with demineralized water. At the start of the experiment, animals of different sizes were allocated either to a fed or a starved group. Amphipods in the starved group were housed individually in a plastic jar (volume: 50 mL) closed with mesh that allowed the exchange of water between the jar and tray. Starved amphipods were deprived of fish food for 14 days, at 10 °C, 16 h:8 h L:D regime. The fed group was given the same feeding regime as before (three times a week with fish food). They were not fed ad libitum, but usually took a few hours to finish the food provided. In addition, we found clear differences in the energy reserves between fed and starved amphipods (Fig. 7, biochemical result) indicating that whether animals were fed or starved induced the intended contrasts. Mortality during the 2 week period was low (< 7%). Following this 2 week period, we measured metabolic rate, heat tolerance and performed biochemical analyses on the amphipods.

In total, 115 amphipods were exposed to starvation conditions, of which 25 were used to measure metabolic rate, 62 for heat tolerance and 28 of them were used for analysis of glycogen, glucose, total fat, total protein and HSP70. The fed group consisted of 120 amphipods, of which 28 were used to measure metabolic rate, 64 for heat tolerance, and 28 for biochemical analyses. An overview of the different measurements performed in this study is shown in Fig. 2.

2.2. Respirometry

Metabolic rates were measured for 25 starved amphipods and 28 control amphipods at 15 °C and 25 °C by analyzing the reduction of dissolved oxygen in the water during stop-flow respirometry. The temperatures are within the natural range of the species and are previously shown to be sufficiently different to induce changes in metabolic rate (Semsar-kazerouni and Verberk, 2018). Glass respiration chambers (two sizes, medium: 4 mL and small: 1 mL) were submerged in a water bath filled with spring water which was continuously filtered by a UV-filter (Sera UV-C-System 5 W) and temperature controlled by means of a Grant R5 water bath with a GP200 pump unit (Grant Instrument Ltd., Cambridge, UK). The water in the chambers was stirred by a glass-coated magnetic stirrer (Loligo, Telemodul20C/40C GmbH). Respiration chambers were fitted with a metal mesh to create a false bottom to prevent contact between the amphipod and the magnetic stirrer. Bacterial background respiration was further minimized by washing all respiratory chambers with ethanol and subsequently rinsing with demi water and drying prior to each experiment. To ensure that the fed amphipods were also in a post-absorptive state, they were deprived of food 1 day before the metabolic rate measurements. Individuals were given 30 min to adjust from the effects of capture and transfer to the respiration chambers before closing the chambers by a lid. Each chamber was fitted with oxygen sensor spot located at the bottom (2 mm in diameter, PreSens, Precision Sensing GmbH). Oxygen concentrations were measured every 15 s using a 10-channel Fiber-Optic Oxygen Meter (OXY-10, PreSens, Precision Sensing GmbH) for a period of 30 min. Seven chambers housed an amphipod and two blanks (without amphipod) which were measured in parallel. A peristaltic pump (Gilson, minipuls 3, Gilson International B.V.) then flushed the chambers for 15 min with spring water from the tub, after which oxygen concentrations were measured once more for 30 min. After two such measurements, the peristaltic pump flushed the chambers continuously and the temperature was increased to the next measurement temperature (25 °C), after which the procedure was repeated. Oxygen concentrations in each chamber were logged from the last 15 min of a measurement cycle (expressed as the slope fitted by a linear regression in μmol min^{-1} L^{-1}). The metabolic 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:

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

After the experiment, the fresh weight of amphipods was determined by gently blotting the animal dry on tissue paper and weighing them to the nearest 0.1 mg. They were then stored in 70% alcohol for species identification (to be sure that all individuals used were indeed *G. fossarum*) and determination of their sex.

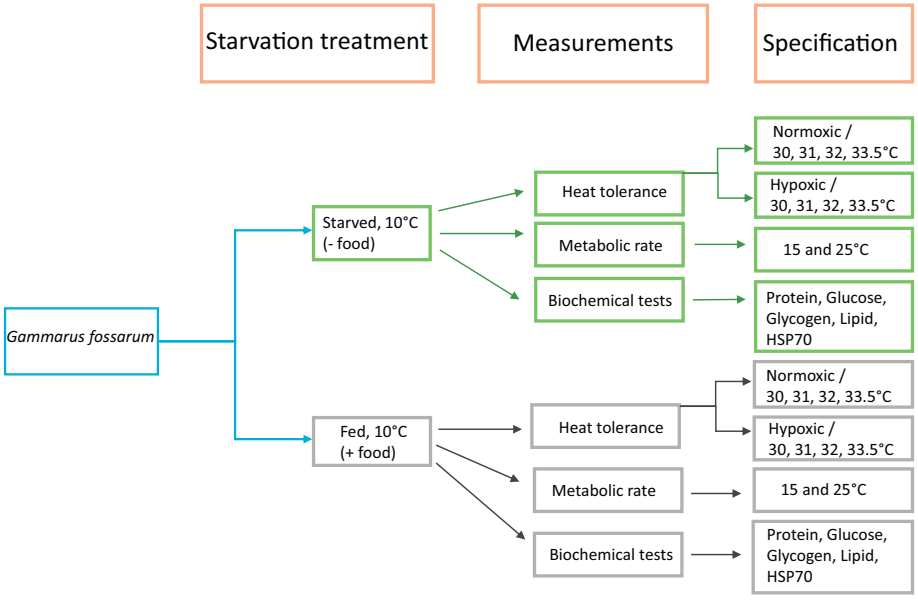

Fig. 2. Schematic illustration of the experimental setup.

2.3. Heat tolerance measurement

The survival time of 64 fed and 62 starved amphipods was tested at 30 °C, 31 °C, 32 °C, and 33.5 °C under normoxic conditions. The highest test temperature which could be tolerated for the shortest duration reflects acute, high-intensity heat stress, whereas the lowest test temperature which could be tolerated for the longest duration reflects prolonged, mild heat stress. An additional 62 amphipods of either the starved or the fed group were exposed to an identical temperature scheme under hypoxic conditions (5 kPa). Oxygen partial pressure (5%) was controlled by two flow controllers (M + W Industries Mass-Stream D-6341-DR), one regulating N_2 flow and one regulating O_2 flow. First animals were transferred to an aerated aquarium with a water temperature of 25 °C for 5 min to minimize the heat shock to the higher test temperatures. After acclimation, the amphipods were individually placed in glass petri dishes that were placed upside down on a semi-transparent nylon mesh so that water was able to flow through the petri dish freely, without any possibility that the amphipod could escape. The amphipods were placed in a temperature-controlled tray (using a Grant Industries TXF200 system) filled with spring water. An UV-filter (Sera UV-C-System 5 W) was active to cleanse the water continuously and the water was aerated to ensure full air-saturation. The survival time of amphipods was recorded with a video recording system (ThorLabs APT ThorCam software version 2.6.7064, ThorLabs Inc. 1.4 Mpixel CCD camera) to allow the time of death to be estimated. This was particularly necessary for the measurements at relatively low temperatures, because the occasionally long survival times (> 8 h) meant that amphipods would die outside office hours. In addition, during office hours, the amphipods were also observed in person, to verify the time of death estimated via the video footage. Every trial included 8 individuals (4 small and 4 large) from either the starved or fed group. We considered an amphipod which had not shown any movement for 2 min as dead. Previous work has shown that while there are different endpoints that could be used (e.g. irregular movement of pleopods used to ventilate the gills or the cessation of movement), these different endpoints where strongly correlated (Verberk et al., 2018). After the experiment, the fresh weight of amphipods was determined by gently blotting the animal dry on tissue paper and weighing them to the nearest 0.1 mg. They were then stored in 70% alcohol for species identification (to be sure that all individuals used were indeed *G. fossarum*) and determination of their sex.

2.4. Biochemical analyses

After 14 days of starvation, 28 starved and 28 fed amphipods were weighed, subsequently frozen in liquid nitrogen and stored at −80 °C and we used them for all biochemical measurements. For homogenization, each frozen amphipod was placed in a round bottom plastic eppendorf (2 mL) with a steel beat (3 mm) and pulverized at 30 Hz for 10 s by use of a grinding mill (Mixer Mill MM 400 (Retsch). Fifteen times (*w/v*) PBS (1 M, pH 7,4) was added to the pulverized amphipods and the samples were homogenized by vortexing for 10 s. Homogenates were kept on ice during all analyses. Remnants of the exoskeleton were removed by 10 min centrifugation at 21000 × *g* (4 °C). Supernatant was transferred to an 1,5 mL eppendorf and 1:100 (*v*/v) Halt^{tm} protease inhibitor cocktail (100×)(ThermoFisher Scientific, #87786) was added and the samples were stored at −20 °C until use for biochemical analyses (such as total lipid, glucose, glycogen, total protein and HSP70 measurements). The biochemical content for glycogen, glucose, total lipid and total protein was calculated according to this formula below:

$$Biochemical\ content\ (\mu g/mg) = \frac{Concentration\left(\frac{\mu g}{\mu L}\right) * Sample\ volume\ (\mu L)}{Mass\ of\ the\ animal\ (mg)}$$

2.4.1. Total protein

Total protein of 28 starved and 28 fed amphipods was measured according to the Bio-Rad protein assay protocol. In short, 5 µL homogenate or albumin standard was mixed with 195 µL 1:5 diluted Bio-Rad Protein Dye reagent concentrate (Bio-Rad, #500–0006) in a 96-wells plate and subsequently incubated for 5 min at 25 °C. The absorbance in each well was measured by platereader (Bio-Rad, Imark^{tm} microplate reader) at 595 nm. All samples and standards were measured in triplo. From the standard curve the protein concentration in the samples was calculated. If necessary, an additional dilution of the samples was performed to reach a concentration of 1 µg protein/µL.

2.4.2. Glucose and glycogen

Glucose of 28 starved and 28 fed amphipods was measured by use of glucose assay reagents in combination with amyloglucosidase treatment. From this data the glycogen concentration was calculated. In short, 25 µL of homogenate (1 µg protein/µL) was mixed with 10 U amyloglucosidase (Sigma A7420, 1 unit/10 µL), supplemented to 100 µL with MQ and incubated for 30 min at 37 °C to convert glycogen into glucose. Each sample was measured twice, once with and once without the addition of amyloglucosidase. For a sample 25 µL of homogenate (1 µg protein/µL) was used and for the standard curve 25 µL of standard (D-(+)-Glucose, Sigma). To both the samples and the standards 75 µL MQ was added. To all samples 200 µL glucose assay reagents (Sigma G3293) was added and subsequently incubated for 20 min at 30 °C. From each sample or standard 100 µL was transferred in triplo to a 384-wells plate and the absorbance was subsequently measured at 340 nm in a platereader (Bio-Rad, Imark^{tm} microplate reader). By use of the standard curve the glucose concentrations were calculated. The concentration of glycogen was calculated as following:

$$Concentration\ of\ glycogen\left(\frac{\mu g}{\mu L}\right) = Reaction with amyloglucosidase enzyme\ (total sugar) - Reaction without amyloglucosidase enzyme\ (glucose)$$

2.4.3. Westernblot of HSP70

The level of HSP70 was measured for 12 starved and 15 fed amphipods. A 10% SDS-PAGE gel was prepared consisting of a resolving gel (10% acrylamide; 0,375 M Tris-HCl pH = 8,8; 0,1% SDS; 0,05% APS; 1:1000 *v*/v TEMED) and stacking gel (4,2% acrylamide; 0,125 M Tris-HCl pH = 6,8; 0,1% SDS; 0,05% APS; 1:700 v/v TEMED). Protein samples were prepared by adding 4× sample buffer ((1×) 62,5 mM Tris-HCl pH = 6,8, 2,5% SDS, 10% glycerol, 0,7 M β-mercaphoethanol, 0,002% bromophenol blue) to 25 µg of protein homogenate and subsequently denatured at 95 °C for 7 min. After cooling to room temperature, the samples were loaded into the gel. Six µL precision plus protein standard was used as a marker (Precision plus protein standards, Bio-Rad, #161–0374). The gel was electrophorated for 30 min at 50 V and subsequently for 1 h at 100 V. Proteins were transferred to a 0,45 µm PVDF membrane (Immobilon®-P Transfer Membrane, #IPVH00005, Merk Millipore Ltd.) by electricity for 2 h at 100 V while being cooled with ice.

Membranes were washed in TBST (10 mM Tris-HCl, 137 mM NaCl; 2,7 mM KCl, pH = 7,4, 0,05% Tween 20) for approximately 5 min and were subsequently blocked with 5% milkpowder in TBST for 1 h. Primary antibody dilution was prepared containing anti-HSP70 (1:1000, ADI-SPA-812 rabbit HSP70/HSP72 polyclonal antibody, Enzo Lifesciences) in TBST and blots were incubated overnight at 4 °C. Unbound primary antibody was removed by washing 3 times with TBST for 10 min. Subsequently blots were incubated in the secondary antibody dilution (TBST, 5% milkpowder, 1:80000, goat anti-rabbit with peroxidase conjugate) (RAM-PO, A9169, Sigma-Aldrich) for 1 h at room temperature. To remove unbound antibody, the blots were washed twice in TBST for 10 min and subsequently twice with TBS for 10 min.

HSP70 protein was visualized by use of the SuperSignal™ West Pico PLUS Chemiluminescent substrate kit (#RH239829A Thermo Scientific) and incubated for 5 min at room temperature. Excess substrate was removed and the blot was exposed to X-ray film (Super RX Fuji Medical X-ray film, #47410 19,236, FujiFilm). Differences in band intensity were quantified by use of Adobe Photoshop CC (2017.1.1 release).

2.4.4. Total lipid

Total lipid of 28 starved and 28 fed amphipods was extracted by adding an equal volume of chloroform/methanol (2:1 *v*/v) to the remaining homogenate. The fat was dissolved in chloroform/methanol by vortexing for 10 s. Separation of the water phase and chloroform phase was done by centrifuging for 10 min at 21000*g* (4 °C) and subsequently the chloroform phase was transferred to a new 1,5 mL eppendorf.

From these samples or from a glyceryl tripalmitin standard 8 μL was mixed with 56 μL sulphuric acid (100%) in a glass tube and heated for 25 min at 150 °C in an incubator (Sanyo dying oven, Mov-112; Sanyo Electric co. ltd, Japan) to facilitate hydrolyzation of the fatty acids. After cooling to room temperature, 64 μL MQ was added to the samples and 74 μL MQ to the standards. This is to compensate for the evaporation of the chloroform in the standards. Thirty μL of sample or standard was transferred in triplicate to a 384-wells plate and absorbance was measured at 340 nm by use of a platereader (Tecan Spark M10 platereader; Tecan Group ltd, Männedorf, Switzerland). Total fat concentration was calculated based on the standard curve.

2.5. Statistical analyses

All analyses were performed in RStudio Version 1.0.136 with standard packages and analyses were considered significant with $P \leq 0{,}05$.

For our analysis of the metabolic rate we fitted a linear mixed effects model lme () from the "nlme" package (Pinheiro et al., 2012), with individual codes as a random factor to account for the non-independence of the two repeated measures from the same individual (Pinheiro and Bates, 2000). The logarithm of oxygen consumption (MO_2) was included as the response variable, and as predictor variables we used the treatment (starved or fed), test temperature, and the body mass of the animals. We also considered whether interactions between treatment (starved or fed) and test temperature led to better model fits based on the AIC values. In a preliminary analysis, we included sequence (first or second measurement) in the model, but as no significant difference was found, we omitted sequence from the final model. Similarly, preliminary analyses also revealed that variation in the time that amphipods were maintained in the lab did not affect metabolic rate ($P = .187$) and we therefore omitted this variable from the final model.

For analyzing the survival data, all survival time measurements were log10 transformed to linearize the relationship with stress temperature (Rezende et al., 2014). We used linear models to test for an effect of starvation and test temperature on survival time. We considered whether interactions between treatments and test temperature led to better model fits based on AIC values. Preliminary analyses showed that the effects of temperature were sometimes better modelled by a non-linear relation. For the sake of consistency, we therefore modelled temperature as a 2nd degree polynomial in all the analyses. In the analyses where we tested whether the TDT curves differed between normoxia and hypoxia we also considered whether interactions between oxygen and test temperature led to better model fits based on AIC values. We also considered the effect of body mass, but found no significant effect and omitted body mass from the final model. Preliminary analyses revealed that variation in the time that amphipods were maintained in the lab did not affect heat tolerance ($P = .961$). As these preliminary analyses also showed a three-way interaction between starvation, test temperature and oxygen, we analyzed starved and fed animals separately.

To test for effects of starvation in the biochemical parameters (glucose, glycogen, lipid, protein and HSP70) linear regression models were used. In our models, we included biochemical parameters as a response variable and we considered treatments and the logarithm of body mass as predictor variables. In all analyses, model assumptions such as normal distribution and homogeneity of variances were assessed visually. In cases where there was doubt whether these test assumptions were met, we tested the robustness of our models. A model was considered robust if the same result was obtained on a subset of the data without those data points that strongly deviated from the test assumptions. Since all amphipods for the biochemical parameters were frozen at the same time point, there was no need to test for an effect of variation in the time that amphipods were maintained in the lab.

3. Results

3.1. Survival

3.1.1. Heat tolerance under normoxic conditions

In both starved and fed animals, survival was strongly affected by test temperature (test temperature: $F = 143.20$; $P < .0001$; Fig. 3; Table 2) with survival time logically declining when heat stress became more intense (Fig. 3; Table 2). The thermal death time curve of the starved group differed from the fed group, as shown by the interaction between test temperature and starvation treatment ($F = 9.27$, $P = .0003$) (Table 2). Compared to starved amphipods, fed amphipods showed better survival under mild, but prolonged heat stress, yet they were (slightly) worse at surviving shorter, intense heat stress (Fig. 3).

3.1.2. Heat tolerance of fed amphipods under both normoxic and hypoxic conditions

In fed amphipods, the thermal death time curve under hypoxia (5 kPa) clearly differed from the normoxia curve ($F = 18.33$, $P < .0001$). The change with hypoxia was similar to the difference observed with starved amphipods (Fig. 4; Table 3): When heat stress was relatively mild, hypoxia reduced the survival of fed amphipods. Surprisingly, hypoxia (slightly) improved the heat tolerance at the highest test temperature.

3.1.3. Heat tolerance of starved amphipods under both normoxic and hypoxic conditions

The thermal death time curve of starved amphipods was decreased under hypoxic conditions compared to the normoxic condition. This decrease in survival was apparent during both mild and intense heat stress (i.e. the lines did not cross as was the case for fed animals), something which is also reflected in the fact that we did not find a

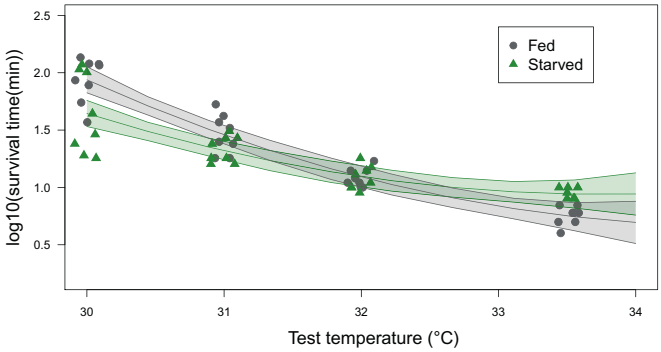

Fig. 3. Heat tolerance expressed as survival time (log10-transformed) of starved (green triangles) and fed (grey circles) amphipods under normoxic condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Anova table for survival time (log10-transformed) in *Gammarus fossarum* based on test temperature, starvation treatment and interaction effects.

Factor	DF	Sum Sq	Mean Sq	*F*-value	*P*-value
Test temperature (2nd polynomial)	2	7.9267	3.9633	143.20	< 0.0001
Starvation treatment	1	0.0557	0.0557	2.01	0.1614
Test temperature (2nd polynomial)*starvation treatment	2	0.5131	0.2565	9.27	0.0003
Residuals	58	1.6053	0.0277		

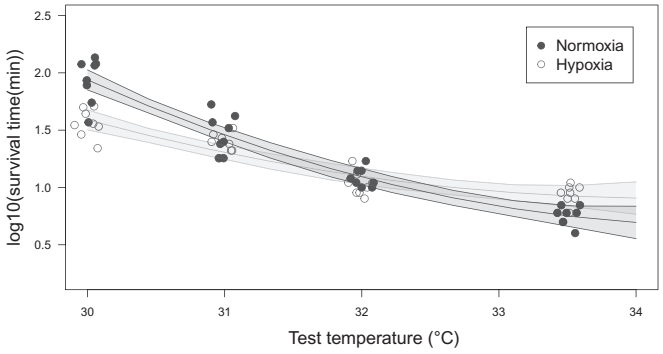

Fig. 4. Heat tolerance of fed amphipods under hypoxic (white circles) and normoxic (grey circles) conditions.

Table 3
Anova table for survival time (log10-transformed) in fed amphipods based on test temperature (modelled as a second degree polynomial), oxygen conditions (both normoxic and hypoxic condition) and their interaction.

Factor	DF	Sum Sq	Mean Sq	*F*-value	*P*- value
Test temperature (2nd polynomial)	2	7.5716	3.7858	231.62	< 0.0001
Oxygen	1	0.1032	0.1032	6.32	0.0148
Test temperature (2nd polynomial)*oxygen	2	0.5990	0.2995	18.33	< 0.0001
Residuals	58	0.9480	0.0163		

Table 4
Anova table for survival time (log-transformed) in starved *Gammarus fossarum* based on test temperature (modelled as a second degree of polynomial), oxygen conditions (both normoxic and hypoxic condition) and their interaction.

Factor	DF	Sum Sq	Mean Sq	*F*-value	*P*-value
Test temperature (2nd polynomial)	2	4.4077	2.20387	77.14	< 0.0001
Oxygen	1	1.5864	1.58639	55.52	< 0.0001
Test temperature (2nd polynomial)*oxygen	2	0.1249	0.06245	2.19	0.122
Residuals	56	1.6000	0.02857		

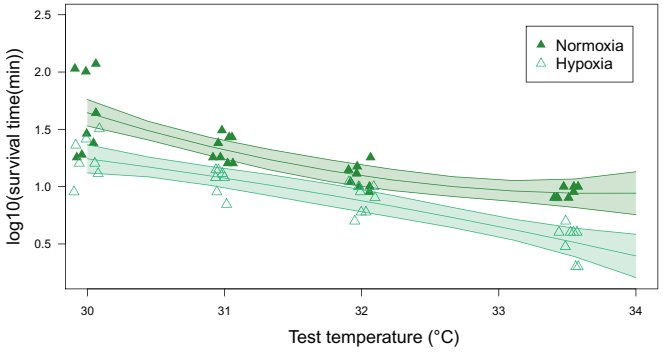

Fig. 5. Heat tolerance of starved amphipods under hypoxic (open triangles) and normoxic (filled triangles) conditions.

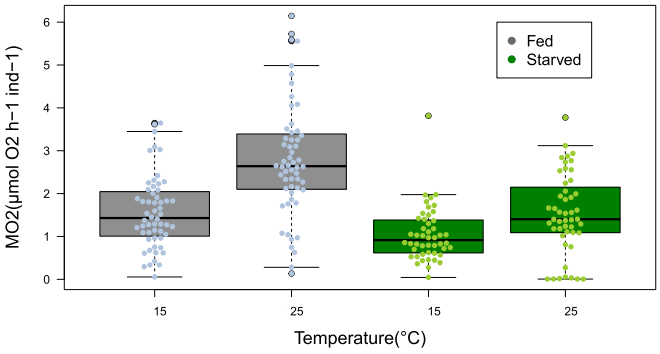

Fig. 6. Individual oxygen consumption rates (µmol O_2/hour) at two test temperatures in starved and fed amphipods.

significant interaction between test temperature and effects of starvation (F = 2.19, P = .122) (Fig. 5; Table 4).

3.2. Metabolic rate

Starvation decreased the rate of oxygen consumption in amphipods (F = 19.22, P = .0001), particularly at the higher temperature of 25 °C (Fig. 6; Table 5). Metabolic rate increased with increasing temperature (F = 5.31, P = .023), and this increase was most notable in fed animals, reflecting the significant interaction between temperature and the starvation treatment (F = 7.30, P = .008). Larger amphipods also displayed higher oxygen consumption rates (F = 9.21, P = .004).

3.3. Biochemical analyses

3.3.1. Total protein, glycogen, glucose and lipid

There were significant effects of the starvation treatment on the energy reserves of the amphipods (Table 6). After two weeks of starvation, a significant decline was observed in glucose and glycogen content (Fig. 7). We also found lower fat levels and protein levels, but these were less pronounced, especially for proteins (Fig. 7). The effect of body mass was also considered in the all biochemical analyses, and only for glucose did we find a size dependency, with larger animals having more glucose (see Table 6).

3.3.2. HSP70 levels

There was no significant effect of starvation (F = 0.56, P = .463) and mass (F = 2.67, P = .115) on HSP70 level in both starved (Mean pixel intensity = 689,377; SD = 660,887) and fed (Mean pixel

Table 5
Anova table for oxygen consumption (log-transformed) as a function of body mass, and the interaction between test temperature and starvation treatment.

Factor	numDF	denDF	*F*-value	*P*- value
Intercept	1	154	444.09	< 0.0001
Test temperature	1	154	5.31	0.023
Starvation treatment	1	49	19.22	0.0001
log10 (mass)	1	49	9.21	0.004
Test temperature* starvation treatment	1	154	7.30	0.008

Table 6
Anova table for glucose (μg/mg), glycogen (μg/mg), fat (μg/mg), and total protein as a function of starvation treatment and mass (mg).

Response variable	Factor	DF	Sum Sq	Mean Sq	*F*-value	*P*-value
Glucose	Starvation treatment	1	22.401	22.4006	21.73	< 0.0001
	Log10 (mass)	1	5.814	5.8140	5.64	0.021
	Residuals	53	54.647	1.0311		
Glycogen	Starvation treatment	1	48.298	48.298	31.75	< 0.0001
	Log10 (mass)	1	5.679	5.679	3.73	0.059
	Residuals	53	80.629	1.521		
Fat	Starvation treatment	1	2179.9	2179.86	7.02	0.011
	Log10 (mass)	1	69.4	69.37	0.22	0.639
	Residuals	53	16,468.7	310.73		
Protein	Starvation treatment	1	370.8	370.76	6.19	0.016
	Log10 (mass)	1	15.5	15.49	0.26	0.613
	Residuals	53	3177.2	59.95		

intensity = 503,355; *SD* = 404,138) amphipods (Table 7).

Table 7
Result of linear regression model on differences in HSP70 as a function of starvation treatment and mass (g).

Factor	DF	Sum Sq	Mean Sq	*F*-value	*P*- value
Starvation treatment	1	5.3343e+10	5.3343e+10	0.56	0.463
log10 (mass)	1	2.5607e+11	2.5607e+11	2.67	0.115
Residual	24	2.3007e+12	9.5862e+10		

4. Discussion

In the present study, we tested if starvation changes patterns in heat tolerance, and if such changes can be explained from modifications in energy metabolism, energy reserves, production of heat shock proteins or susceptibility to oxygen limitation. As these modifications are unlikely to operate on the same timescale, we included the effect of time in our study by studying tolerance of ectotherms to different levels of heat stress intensity. The ability of ectotherms to cope with heat stress depends not only on the intensity but also on the duration of exposure to heat stress (Rezende et al., 2014), something we previously demonstrated for our study species (Semsar-kazerouni and Verberk, 2018). Thus, rather than employing a single temperature, we investigate heat tolerance across conditions ranging from acute, high-intensity heat stress to more prolonged survival under milder levels of heat stress.

We observed changes in heat tolerance following starvation, with starved animals having improved survival at the highest test temperature (reflecting acute, high-intensity heat stress), but not at the lower test temperature (reflecting prolonged, mild heat stress) when compared to fed animals (Fig. 3). An improvement in heat tolerance could result from increased levels of heat shock proteins, as previous work has demonstrated that stress resulting from starvation can stimulate the production of heat shock proteins (Cara et al., 2005; Nguyen et al., 2017; Yengkokpam et al., 2008). This mechanism would be most likely to operate at fast time scales (minutes) and in response to high intensity heat stress where protein inactivation and denaturation is more likely. However, we did not observe significant differences in HSP70 levels between the starved group and the fed group. Possibly, production of heat shock proteins was not upregulated because the starvation period was not sufficiently long (i.e. 14 days). Previously, Hervant et al. (1999) showed that this species can tolerate food limitation for up to 28 days. Another explanation could be that starvation induced metabolic downregulation, which reduces protein synthesis rates, including the production of HSP70. An alternative hypothesis is that oxygen limitation sets heat tolerance (Pörtner, 2010; Verberk et al., 2016). According

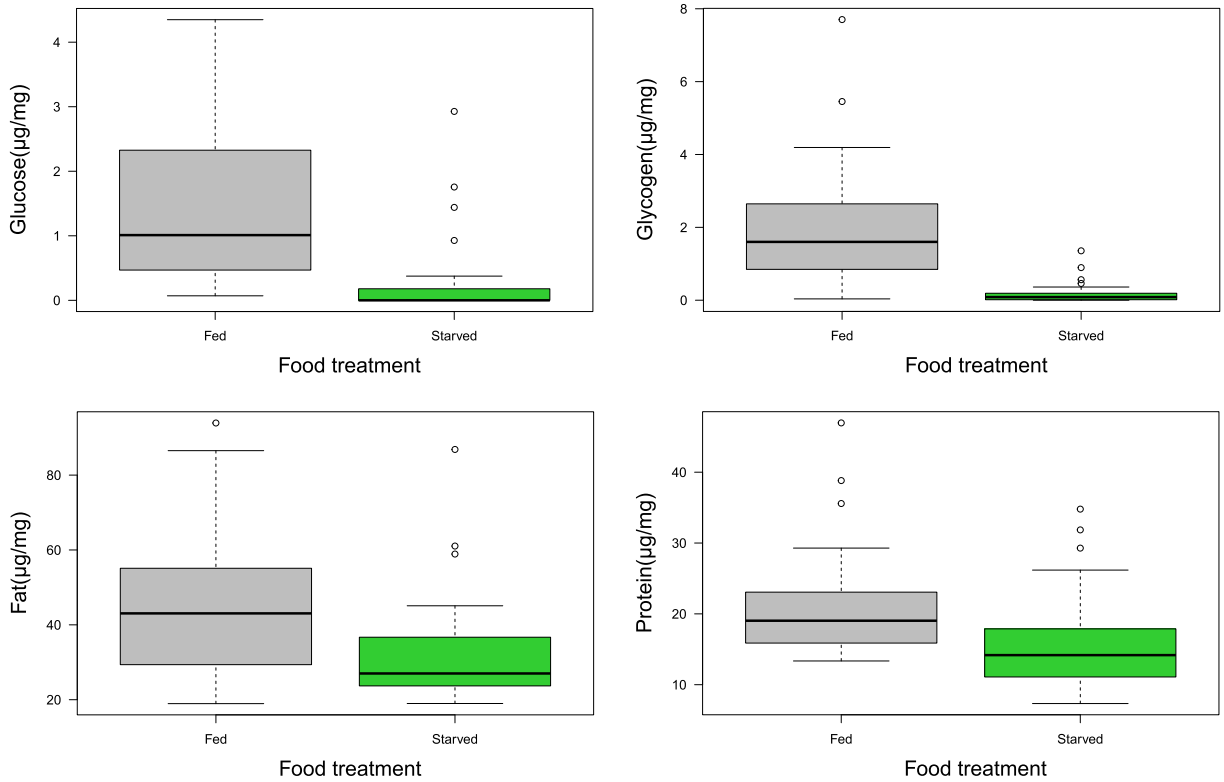

Fig. 7. Levels of energy storage: glucose (μg/mg), glycogen (μg/mg), fat (μg/mg), and total protein (μg/mg) of starved (green) and fed (grey) amphipods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to this hypothesis, the downregulation of metabolic rate could lead to an improved heat tolerance of starved animals by lowering their oxygen requirements. Previous studies have shown reductions in oxidative metabolism following starvation (Hervant, 2012; McCue, 2010). Our work likewise showed that starved animals had reduced their oxygen consumption compared to fed animals, and this difference was most pronounced at the higher test temperature of 25 °C (Fig. 6). Therefore, the improved survival we observed under high-intensity heat stress could be explained from amphipods entering a hypometabolic state upon starvation. Still, for mild, prolonged heat stress this hypometabolic state appeared to have little effect.

The impaired tolerance to mild, prolonged heat stress in starved amphipods could be caused by the depletion of their energy reserves. Starved amphipods had significantly lower reserves of carbohydrates (glucose, glycogen) and lipids (Fig. 7), suggesting these were used as metabolic fuel before catabolizing protein. Similar responses to starvation have been reported for other ectotherms (Hervant et al., 1999). Vinagre and Chung (2016) also found that the level of triglycerides (in the hepatopancreas) and glycogen reduced during starvation in Atlantic ghost crab. The depletion of energy reserves impairs the ability of starved amphipods to generate energy, which is necessary to meet the elevated energy demands during heat stress (resulting from enhanced energetic costs e.g. protein synthesis and ion transport).

To test whether heat tolerance was limited by a lack of oxygen, we assessed the survival of heat stress under hypoxic conditions and compared this to the survival under normoxic conditions. We observed clear effects of hypoxia, which generally impaired heat tolerance. Importantly, the effect of hypoxia differed between starved and fed amphipods on a temporal scale. Under prolonged, mild heat stress, hypoxia impaired the ability of both fed and starved amphipods to tolerate heat. This suggests that hypoxia impairs the ability of amphipods to meet the elevated energy demands during heat stress. Given the ~15 fold higher ATP production associated with aerobic metabolism compared to anaerobic metabolism, the lower heat tolerance of amphipods under mild, prolonged heat stress suggests a limitation in oxygen supply under hypoxic conditions. Anaerobic metabolism may supplement ATP production, also under normoxic conditions, but rapidly depletes glucose stores, explaining the difference between starved and fed animals in their tolerance to low-intensity heat. Indeed, other studies have shown that energy reserves may play a significant role in thermal tolerance (Manenti et al., 2018; Colinet et al., 2006). Colinet et al. (2006) revealed that energy reserves in *Aphidius colemani* are critical for survival during thermal stress. Our findings suggest that energy reserves play an important role in dealing with mild, prolonged heat stress, while starvation actually improved thermal tolerance under acute, high-intensity heat stress, but only under normoxic conditions. Hypoxia impaired tolerance to acute, high-intensity heat stress in starved animals, indicating they depend on a continuous and adequate flux of oxygen from their environment. Depletion of energy reserves in starved amphipods reduced the ability to generate ATP via anaerobic metabolism, and aerobic metabolism was used to meet the energy demands in their hypometabolic state. In contrast to starved animals, tolerance to high-intensity heat stress in fed animals was not impaired under hypoxia and fed animals even appeared more tolerant to high-intensity heat under hypoxia (Fig. 4). This suggests that fed animals are able to rely more on anaerobic metabolism to facilitate ATP production under hypoxic conditions. Anaerobic metabolism is less efficient in generating ATP but it will help maintain energy status, at least on the short term (Pörtner, 2010). A metabolomics study showed evidence of the upregulation of sugar based metabolic pathways to fuel anaerobic metabolism in heat stressed stoneflies (Verberk et al., 2013). The increase in sugar based metabolites (pentitolphosphates) occurred in response to heat stress and this increase was stronger under hypoxia. Therefore, hypoxia may have augmented the switch to anaerobic metabolism thus explaining why hence fed animals could better tolerate high-intensity heat stress under hypoxia. Using energy reserves to fuel anaerobic metabolism is a feasible strategy but only in the short term and only for fed animals.

Observed differences between acute, intense heat stress and mild, prolonged heat stress indicate that strategies for tolerating heat stress are likely time dependent. This time dependency could help resolve the contradicting results of previous studies, which reported either positive or negative effect of starvation on heat tolerance. A direct comparison of these studies is challenging, because of differences in the choice of species, the starvation protocols used and types of how heat tolerance was measured. However, it is possible that positive effects of starvation are more likely on shorter timescales, whereas negative effects are more likely on longer timescales. For instance, Manenti et al. (2018) observed a significant reduction in heat tolerance for starved flies in their slowest ramping rate trail compared to the fastest ramping rate trial. The improvement in heat tolerance found by Mitchell et al. (2017) who studied thermal tolerance on very short timescales would also fit with this idea (see Table 1). However, other studies employ longer timescales of around an hour yet still report positive effects of starvation (Mutamiswa et al., 2018; Gotcha et al., 2018; DeVries et al., 2016). These timescales could arguably be classified as short and a more definitive test of this idea would be to study starvation effects in these species over longer time scales.

In conclusion, we observed both beneficial and detrimental effects of starvation on the heat tolerance of amphipods. Increases in heat tolerance are likely caused by a lower energy demand in starved animals, whereas decreases might be related to depletion of energy stores. Our findings on heat tolerance patterns under normoxia and hypoxia suggest that fed amphipods rely more on anaerobic metabolism to maintain energy status during heat stress, especially when heat stress is intense. However, for starved amphipods aerobic metabolism appears more important. Contrasting results in the literature on the consequences of starvation for heat tolerance could arise from the different mechanisms that link starvation and heat tolerance and the time scale on which they operate.

Data Availability

All data files are available from the DANS EASY archive (DOI: https://doi.org/10.17026/dans-28c-rm2b).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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